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19. ABSTRACT

The general aim of this research has been to understand the electrophysiological properties and synaptic mechanisms of suprachiasmatic nucleus (SCN) neurons. Our earlier experiments included sharp-intracellular-electrode analyses of amino-acid-mediated synaptic transmission and intrinsic membrane properties, focussing on the degree to which SCN neurons are homogenous or heterogenous. This work showed that GABA (in addition to glutamate) plays a critical role in fast synaptic transmission in the SCN, and that SCN neurons are not homogenous in terms of their electrophysiological properties, although they could not be grouped into distinct neuron classes. More recently, multiple-unit extracellular recordings have shown synchronous bursts of action potentials in the SCN in low [Ca^{2+}] solutions containing amino-acid-receptor antagonists demonstrated to block chemical synapses, thus suggesting that SCN neurons can communicate through nonsynaptic mechanisms. Our more recent studies using whole-cell patch-clamp techniques in the thin-slice preparation have shown evidence for local GABA-ergic communication among SCN neurons, and have begun to define the different types of K^+ currents present in SCN neurons. We are also in the final stages of completing several studies on the supraoptic and paraventricular nuclei and on neurons from the preoptic area of the hypothalamus, thus providing a framework for comparing neurons in the SCN with other hypothalamic areas. This research is now focussing largely on local synaptic circuits and K^+ currents, and how they regulate the electrical behavior of SCN neurons.

TABLE OF CONTENTS

1. RESEARCH OBJECTIVES

The primary aim of this research program has been to test hypotheses concerning the electrophysiological properties and synaptic mechanisms of suprachiasmatic nucleus (SCN) neurons. Our previous work involved intracellular recording with sharp electrodes and traditional hypothalamic slices; this then evolved into extracellular recordings from populations of neurons to study nonsynaptic mechanisms; and more recently has focussed on using whole-cell patch-clamp recording techniques in the thin slice preparation. This latter research, which is currently underway, has focussed on local GABA-mediated synaptic circuits and on identifying the different types of K^+ currents in SCN neurons. In addition, we have completed several electrophysiological studies on the supraoptic and paraventricular nuclei and on the preoptic area, which has provided the basis for allowing us to make comparisons between the SCN and other regions of the hypothalamus.

2. STATUS OF RESEARCH

A. Suprachiasmatic nucleus (SCN)

(i) Intracellular sharp-electrode electrophysiology

(a) Gamma-amino-butyric acid (GABA)

We have completed the second of a pair of electrophysiological studies aimed at testing the hypothesis that GABA and glutamate are responsible for inhibitory and excitatory postsynaptic potentials (IPSPs and EPSPs). We previously published a paper providing evidence that glutamate is responsible for all detectable EPSPs arising from optic nerve stimulation and from other sources. We have now published a second paper showing that spontaneous and evoked fast IPSPs are mediated by GABA acting on a $GABA_A$ receptor. We found that the IPSP reversal potential was approximately -75 mV, that the IPSPs were positive-going when recorded with KCl electrodes, and that the IPSPs were blocked by bicuculline (a $GABA_A$ receptor antagonist). We found no evidence for slow IPSPs in SCN neurons. These data strongly support the hypothesis that SCN neurons receive extensive GABA-ergic input, and that $GABA_A$ receptors cause an increase in chloride conductance to generate fast IPSPs in SCN neurons. The paper on GABA-mediated synaptic mechanisms was published in the *Journal of Physiology* (Kim and Dudek, 1992).

(b) Membrane properties

Intracellular recordings were obtained from SCN neurons to determine whether they had homogenous or heterogenous electrophysiological properties. Most of our efforts were aimed at recording from neurons that received retinal input. An extensive quantitative analysis was undertaken of resting potential, input resistance, action potentials, and hyperpolarizing afterpotentials; these properties were similar across SCN neurons. We found that some neurons showed time- and

voltage-dependent inward rectification, and some of them had low threshold Ca^{2+} spikes. We also found that firing pattern (i.e., regular versus irregular) was directly related to firing rate rather than neuron type. These data have been published in a paper in the *Journal of Physiology* (Kim and Dudek, 1993), and the membrane properties are under further analysis using whole-cell patch-clamp recordings.

(iii) Non-chemical-synaptic mechanisms of synchronization

It has been widely accepted that communication between neurons in the hypothalamus is mediated primarily by Ca^{2+}-dependent synaptic transmission. Simultaneous extracellular recordings of neuronal action potentials in the SCN demonstrated synchronized bursts of action potentials in Ca^{2+}-free medium, which blocks chemical synaptic transmission and increases membrane excitability. These periodic bursts of synchronized action potentials occurred across a large population of SCN neurons and persisted in the presence of NMDA, non-NMDA and $GABA_A$ receptor antagonists. Whole-cell recordings confirmed that postsynaptic potentials were blocked in this solution. These data provide strong evidence that mechanisms other than Ca^{2+}-dependent synaptic transmission can synchronize neurons in the mammalian SCN. A paper summarizing these results has been published in the *Proceedings of the National Academy of Sciences* (Bouskila and Dudek, 1993).

(iv) Whole-cell patch-clamp recordings

The experiments described above were all initiated before the renewal of our grant proposal, and they all used traditional 500 μm thick hypothalamic slices and primarily sharp intracellular recording techniques. The experiments described below represent completely new experiments, using the thin-slice technique (i.e., 100-200 μm slices) where SCN neurons were viewed directly with Nomarski optics on an upright compound microscope. Although firm conclusions are not yet available, this approach has been extremely productive, and two extensive studies are underway. Our preliminary results will be described in brief below.

(a) Local inhibitory circuits

Whole-cell patch-clamp recordings of inhibitory postsynaptic currents (IPSPs), combined with glutamate microstimulation techniques, are being used to study local synaptic connections within the SCN. Microapplication of glutamate, as opposed to traditional electrical stimulation, allows one to activate neurons within the SCN independent of axons of passage. Use of the thin-slice technique and direct visualization of the SCN has permitted us to microapply extremely small volumes of glutamate within specific regions of the SCN. Intracellular staining techniques, with biocytin inside the patch pipette, has allowed us to not only confirm conclusively that the neurons are in the SCN, but to also identify which part of the SCN contains the recorded neurons. Although quantitative data are not yet available, we have recorded from over two dozen SCN neurons while glutamate was microapplied near the recording site in the SCN. Several neurons showed

increases in the amplitude and frequency of IPSCs concomitant with glutamate microapplication. These IPSCs were blocked by bath application of bicuculline (10 μM). An abstract on this work will be presented at the annual meeting of the Society for Neuroscience (Strecker and Dudek, 1994). These experiments, although preliminary, strongly suggest that SCN neurons provide inhibitory synaptic input to other SCN neurons. Ongoing experiments are aimed at confirming these results, and future studies will involve experiments with tetrodotoxin aimed at determining whether these events are dependent on Na^+-mediated action potentials.

(b) Potassium (K^+) currents

Preliminary experiments have been performed on K^+ currents in Ca^{2+}-free solutions. We have identified a transient outward K^+ current in these neurons, and an extensive voltage-clamp protocol is being used with K^+ current antagonists (i.e., 4-aminopyridine and tetraethylammonium). In a few months we expect that more solid conclusions will be available.

B. Other hypothalamic regions

Virtually all of the data described below was summarized in the Progress Report of our grant application. A summary will be repeated below, since final revisions of manuscripts and publications of these papers occurred during the last budget year.

(i) Paraventricular nucleus (PVN)

The purpose of these experiments was to test the hypothesis that neurons in the paraventricular nucleus (PVN) receive GABA-mediated inhibitory synaptic input from neurons in the general region of PVN. Intracellular recordings were obtained from neurons in and around the PVN, and glutamate microdrops were applied to slices dorsal, lateral, and ventral PVN to selectively activate local presynaptic neurons. Local glutamate microapplication increased the frequency and amplitude of IPSPs, and these IPSPs were blocked by bath application of the $GABA_A$ receptor antagonist bicuculline. These data provide evidence that local synaptic circuits are primarily inhibitory and supplied by perinuclear GABA-ergic neurons. Furthermore, these data suggest that magnocellular and parvocellular subpopulations in the PVN receive these local inhibitory synaptic inputs. These data suggest that local inhibitory neurons provide feedforward inhibition for excitatory inputs from other brain regions. This research has been published in the *Journal of Physiology* (Tasker and Dudek, 1993).

(ii) Supraoptic nucleus (SON)

Patch-clamp recording techniques allowed us to test more rigorously the hypothesis that GABA and glutamate mediate excitatory and inhibitory postsynaptic currents in the supraoptic nucleus (SON). A quantitative analysis of EPSCs and IPSCs showed that these events were blocked by the non-NMDA

receptor antagonist CNQX, and the IPSCs were blocked by the $GABA_A$ receptor antagonist bicuculline. These results suggest that in the in vitro slice preparation, glutamate mediates all the spontaneous EPSCs in magnocellular neurosecretory cells at resting potential by acting primarily on AMPA/kainate type receptors (i.e., non-NMDA receptors), and that activation of $GABA_A$ receptors mediates most if not all IPSCs. The results of this research were published in the *Journal of Neuroscience* (Wuarin and Dudek, 1993).

(iii) Preoptic area

A series of experiments have been performed with both sharp intracellular and whole-cell patch-clamp recordings in the preoptic area of the hypothalamus. The initial sharp intracellular recordings were correlated with intracellular staining data using biocytin. Even though the intracellular staining showed that recordings were obtained from neurons of diverse morphology, nearly all the neurons had similar electrophysiological properties. In particular, low-threshold Ca^{2+} spikes and linear or nearly linear current-voltage relations were obtained in virtually all neurons. Most neurons had fast EPSPs and IPSPs, and the fast IPSPs were blocked by bicuculline and had a reversal potential near the Cl^- equilibrium potential. This research has been published in the *Journal of Comparative Neurology* (Hoffman, Kim, Gorski, and Dudek, 1994).

More recent studies using whole-cell patch-clamp recordings have shown that spontaneous EPSPs and IPSCs were blocked by bath application of CNQX and bicuculline, providing further evidence that glutamate and GABA mediate virtually all of the fast synaptic currents in the medial preoptic area. This latter paper has tentatively been accepted, pending revisions, in *Brain Research* (Hoffman, Wuarin, and Dudek, submitted).

Two additional review articles have been published in refereed journals (Van den Pol and Dudek, 1993, *Neuroscience* and Dudek, et al., 1993, *Journal of Biological Rhythms*).

In conclusion, a series of electrophysiological studies using 500 μm thick hypothalamic slices and a variety of the electrophysiological techniques on SCN neurons have been published. Another series of experiments on SCN neurons using whole-cell patch-clamp recordings in thin hypothalamic slices are currently underway. Finally, we have published several papers on hypothalamic electrophysiology from other nuclei in the hypothalamus, besides the SCN. Reprints and preprints of these papers are enclosed.

3. PUBLICATIONS

Refereed Publications

Kim, Y.I. and Dudek, F.E. (1992) Intracellular electrophysiological study of suprachiasmatic nucleus neurones in rodents: inhibitory synaptic mechanisms. J. Physiol., London 458:247-260 (reprint enclosed).

Kim, Y.I. and Dudek, F.E. (1993) Membrane properties of rat suprachiasmatic nucleus neurons receiving optic nerve input. J. Physiol., London 464:229-243 (reprint enclosed).

Bouskila, Y. and Dudek, F.E. (1993) Neuronal synchronization without active calcium-dependent synaptic transmission in the hypothalamus. Proc. Nat. Acad. Sci. USA 90:3207-3210 (reprint enclosed).

Wuarin, J.P. and Dudek, F.E. (1993) Patch clamp analysis of spontaneous synaptic currents in supraoptic neuroendocrine cells of the rat hypothalamus. J. Neurosci. 13:2323-2331 (reprint enclosed).

Tasker, J.G. and Dudek, F.E. (1993) Local inhibitory synaptic inputs to neurones of the paraventricular nucleus in slices of rat hypothalamus. J. Physiol., London 469:179-192 (reprint enclosed).

Dudek, F.E., Kim, Y.I. and Bouskila, Y. (1993) Electrophysiology of the suprachiasmatic nucleus: synaptic transmission, membrane properties, and neuronal synchronization. J. Biol. Rhyth. 8(Supplement):S33-S37 (invited, refereed review, reprint enclosed).

van den Pol, A.N. and Dudek, F.E. (1993) Cellular communication in the biological clock, the suprachiasmatic nucleus. Neuroscience 56:793-811 (reprint enclosed).

Hoffman, N.W., Kim, Y.I., Gorski, R.A. and Dudek, F.E. (1994) Homogeneity of electrophysiological properties in different neuronal subtypes in medial preoptic slices containing the sexually dimorphic nucleus of the rat. J. Comp. Neurol. 345:396-408 (reprint enclosed).

Manuscripts submitted

Hoffman, N.W., Wuarin, J.P. and Dudek, F.E. Whole-cell patch-clamp recordings of spontaneous synaptic currents in medial preoptic neurons in rat hypothalamic slices: mediation by amino acid transmitters. Brain Res. (accepted, pending revisions).

Chapters (non-refereed)

Bouskila, Y., Dudek, F.E. and Strecker, J. Cellular mechanisms of circadian function in the SCN. Handbook of Behavioral Neurobiology -- Circadian Clocks, Ed. by J.S. Takahashi, F.W. Turek and R.Y. Moore (submitted).

Strecker, J.G., Bouskila, Y. and Dudek, F.E. Physiological properties of suprachiasmatic neurons. Seminars in the Neurosciences (in preparation).

Abstracts

Bouskila, Y. and Dudek, F.E. (1993) Non-chemical synaptic mechanisms synchronize neuronal activity in the suprachiasmatic nucleus (SCN). Sleep Research 22:613.

Bouskila, Y. and Dudek, F.E. (1993) Whole-cell patch-clamp analysis of neuronal excitability in the suprachiasmatic nucleus (SCN). Soc. Neurosci. Abstr. 19:1613, #662.1.

Strecker, G.J. and Dudek, F.E. (1994) Local synaptic circuits in the rat suprachiasmatic nucleus. Soc. Neurosci. Abstr. (submitted).

4. PROFESSIONAL PERSONNEL

Mr. Yona Bouskila
Dr. Neil H. Hoffman
Dr. Yang I. Kim
Dr. Jean-Pierre Wuarin
Dr. G. Joseph Strecker

5. INTERACTIONS

On March 13-17, 1993 I presented the results of our SCN experiments at the International Conference on the Cellular Consequences of Sleep in Maui, Hawaii. The title of the presentation was "Electrophysiology of the Suprachiasmatic Nucleus: Synaptic Transmission, Membrane Properties, and Neuronal Synchronization" (symposium organized by Dr. J. Miller). Mr. Yona Bouskila presented an abstract on his data subsequently published in the *Proceedings of the National Academy of Sciences*.

On June 5, 1993, I participated in a workshop on the "Suprachiasmatic Nucleus as an in vitro model System" at the University of Virginia in Charlottesville, Virginia (organized by Dr. G. D. Block).

Both of these conferences were attended by several investigators supported by the Air Force Office of Scientific Research.

6. NEW DISCOVERIES, INVENTIONS OR PATENT DISCLOSURES--none, other than the research findings described below (see attached patent report).

7. OTHER STATEMENTS

During the next year we will continue to study local synaptic interactions, using glutamate microstimulation and whole-cell patch-clamp recordings. We will also continue our analysis of K^+ currents. We have purchased and recently assembled all of the imaging equipment, and we are prepared to initiate this line of investigation over the next few months. Therefore, considerable progress is being made in several areas outlined in our grant proposal.

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on Reverse Side.)

FORM APPROVED
OMB NO. 0704-0086

1a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (Check one)
Colorado State University	F49620-93-1-0302	Same	Same	[X] INTERIM [ ] FINAL
b. ADDRESS (include Zip Code)	d. AWARD DATE (YYMMDD)	b. ADDRESS (include Zip Code)	d. AWARD DATE (YYMMDD)	4. REPORTING PERIOD (YYMMDD) FROM 7/1/93
Office of Sponsored Programs Fort Collins, CO 80523	7/1/93	Same	Same	TO 6//30/94

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None", so state)*

a. NAME OF INVENTOR(S) (Last, First, M.I.)	b. TITLE OF INVENTION(S)	c. DISCLOSURE NO., PATENT APPLICATION SERIAL NO. OR PATENT NO.	d. ELECTION TO FILE PATENT APPLICATIONS				e. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER	
			UNITED STATES		FOREIGN			
			YES	NO	YES	NO	YES	NO
Dudek, F. Edward	NONE	NONE						

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR.		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
i. NAME OF INVENTOR (Last, First, M.I.)	i. NAME OF INVENTOR (Last, First, M.I.)	i. TITLE OF INVENTION	ii. FOREIGN COUNTRIES OF PATENT APPLICATION
ii. NAME OF EMPLOYER	ii. NAME OF EMPLOYER		
iii. ADDRESS OF EMPLOYER (include Zip Code)	iii. ADDRESS OF EMPLOYER (include Zip Code)		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR. *(If "None", so state)*

a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS (include Zip Code)	c. SUBCONTRACT NO.(S)	d. DAR "PATENT RIGHTS"		e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	f. SUBCONTRACT DATES (YYMMDD)	
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SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if [ ] Small Business or [ ] Non Profit Organization.)* (Check appropriate box)

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, M.I.) Dudek, F. Edward

b. TITLE Professor and Chair

c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

Edward Dudek — 8/1/94

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Journal of Biological Rhythms, Vol. 8, Supplement, 1993, pp. S33–S37

Electrophysiology of the Suprachiasmatic Nucleus: Synaptic Transmission, Membrane Properties, and Neuronal Synchronization

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Abstract Knowledge of the neuronal membrane properties and synaptic physiology of the suprachiasmatic nucleus (SCN) is critical for an understanding of the cellular basis of circadian rhythms in mammals. The hypothalamic slice preparation from rodents and a combination of electrophysiological techniques (i.e., extracellular single- and multiple-unit recording, intracellular recording, and whole-cell patch clamp) were used to study (1) the role of excitatory and inhibitory amino acids (i.e., glutamate and γ-aminobutyric acid [GABA] in synaptic transmission, (2) the membrane properties of SCN neurons, and (3) the mechanisms of neuronal synchronization. Antagonists for *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors blocked excitatory postsynaptic potentials (EPSPs) evoked by stimulation of the optic nerve or other sites when cells were depolarized or at rest, respectively. Bicuculline blocked inhibitory postsynaptic potentials (IPSPs) that were evoked by local stimulation or that occurred spontaneously. The IPSP reversal potential was near the Cl^- equilibrium potential, and was shifted to depolarized levels by raising intracellular [Cl^-]. Thus, glutamate and GABA appear to mediate fast excitatory and inhibitory synaptic transmission in the SCN. Some SCN neurons, but not all of them, had low-threshold Ca^{2+} spikes and time-dependent inward rectification, thus indicating that the electrical properties of SCN neurons are not homogenous. Neurons with a firing rate of >6 Hz had a regular pattern, and neurons with a rate of <4 Hz had an irregular pattern; since both the firing rate and pattern could be modified with injected currents, SCN neurons with different firing patterns are unlikely to represent distinct classes of cells. Synchronous bursts of action potentials occurred in the SCN after chemical synapses were blocked with [Ca^{2+}]-free solutions and with amino acid transmitter antagonists, which indicates that synchronous neuronal activity can occur in the SCN without active chemical synapses and suggests that a different mechanism of communication exists in the SCN. Future *in vitro* electrophysiological experiments should provide an explanation of how neurotransmitters, local neuronal circuits, and intrinsic membrane properties regulate the electrical activity of SCN neurons during the circadian rhythm.

Key words circadian rhythm, glutamate, GABA, membrane properties, synaptic, nonsynaptic, synchronization

Although considerable information is available concerning the important role of the suprachiasmatic nucleus (SCN) in the generation of circadian rhythms (Klein et al., 1991), very little is known about the electrophysiological properties of SCN neurons at the cellular and membrane levels. The relatively small size (~ 10 μm) of SCN neurons has made it difficult

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to record intracellularly with traditional sharp-electrode techniques, even in slice preparations. The few available studies that used intracellular recordings in hypothalamic slices (Wheal and Thomson, 1984; Sugimori et al., 1986; Thomson and West, 1990) did provide fundamental information on the physiological properties of SCN neurons, but they left many unanswered questions about the transmitters responsible for excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs), the homogeneity of the membrane properties, and the cellular mechanisms of synchronization of electrical activity. The experiments described below were aimed at (1) identifying the receptors mediating EPSPs and IPSPs; (2) identifying the intrinsic electrical properties of SCN neurons, with an emphasis on the presence of low-threshold Ca^{2+} spikes and time-dependent inward rectification, and on the issue of the homogeneity–heterogeneity of SCN electrical properties; and (3) determining whether Ca^{2+}-dependent chemical synaptic transmission is required for the synchronization of electrical activity in the SCN.

EXCITATORY AMINO ACIDS

The role of excitatory amino acid receptors in the generation of excitatory synaptic responses to optic nerve stimulation was studied in horizontal and parasagittal hypothalamic slices from rats and guinea pigs (Kim and Dudek, 1991). Activation of retinal input evoked EPSPs in SCN neurons. The antagonist for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate-type receptors, 6,7-dinitroquinoxaline-2,3-dione (DNQX, 1–10 μM), blocked EPSPs in a concentration-dependent and reversible manner. The selective *N*-methyl-D-aspartate (NMDA) receptor antagonist, D,L-2-amino-5-phosphonopentanoic acid (AP5, 50–100 μM), did not significantly and consistently affect the EPSPs at resting or hyperpolarized membrane potentials; however, when SCN neurons were depolarized, AP5 blocked or depressed a slow component of the EPSPs. Similar data were obtained for spontaneous EPSPs and EPSPs to stimulation at other sites. These results demonstrate that both non-NMDA and NMDA receptors mediate excitatory synaptic transmission both from retinal input and from other central nervous system sites, and that NMDA receptors contribute to EPSPs in depolarized SCN neurons, in agreement with the removal of Mg^{2+} block during depolarization.

γ-AMINOBUTYRIC ACID

The mechanisms of inhibitory synaptic transmission were also studied (Kim and Dudek, 1992). Electrical stimulation dorsocaudal to the SCN evoked fast IPSPs in most neurons, and spontaneous IPSPs were present in every neuron. Spontaneous and evoked IPSPs were hyperpolarizing at resting potential and had a reversal potential of ~ -75 mV. With KCl electrodes, the IPSPs were positive-going. The IPSPs were blocked by bicuculline, a γ-aminobutyric $acid_A$ ($GABA_A$) receptor antagonist. Bicuculline-resistant hyperpolarizing potentials, similar in time course to the fast IPSPs, occurred spontaneously in some cells and could be evoked by electrical stimulation of the optic nerve or a dorsocaudal site. A fast prepotential (i.e., a partial action potential) always preceded these hyperpolarizing potentials, and injection of hyperpolarizing currents blocked these events, thus indicating that they were not synaptic in origin. The properties of these hyperpolarizations indicate that they represent the passive reflection of the hyperpolarizing afterpotentials from blocked

action potentials (e.g., dendritic spikes or electrotonic coupling potentials). No slow IPSPs were detected in SCN neurons. Therefore, SCN neurons receive extensive GABA-ergic input; $GABA_A$ receptors and an increase in Cl^- conductance mediate these IPSPs.

MEMBRANE PROPERTIES

Electrophysiological properties were studied to determine whether SCN neurons are homogeneous or heterogeneous, and whether distinct classes of neurons could be identified (Kim and Dudek, 1993). The experiments focused on the subpopulation of neurons that demonstrably received retinal input, as determined by recording short-latency EPSPs to optic nerve stimulation (Kim and Dudek, 1991). Individual action potentials were relatively short in duration, and were followed by a pronounced hyperpolarizing afterpotential. Spike inactivation, spike broadening, and frequency accommodation occurred consistently during depolarizing current pulses, and an afterhyperpolarization routinely followed a burst of action potentials. With sharp electrodes, the membrane time constant of these neurons ranged from 7 to 21 msec (mean = 11.4 ± 0.7 msec), and input resistance was 105 to 626 MΩ (mean = 301 ± 23 MΩ). Although there was some variability in electrical properties, no distinct groups were found when analyses were made across the neuronal population. Some neurons did show slight time- and voltage-dependent inward rectification, and these neurons had a higher spontaneous firing rate and were more excitable. Some neurons also had low-threshold Ca^{2+} spikes, although other neurons clearly lacked them. Most neurons fired spontaneously; those neurons with a firing rate of >6 Hz had a regular firing pattern, whereas neurons that fired at <4 Hz had an irregular pattern, as reported previously by Thomson et al. (1984). Altering the firing rate with injected current changed the firing pattern. These results suggest that (1) SCN neurons receiving optic nerve input are not electrophysiologically homogeneous, but they do not yet appear to form distinct classes of electrophysiological cell types; (2) time-dependent inward rectification and the capacity to generate low-threshold Ca^{2+} spikes are limited to only a subpopulation of SCN neurons; (3) time-dependent inward rectification is associated with an increased spontaneous firing rate and excitability; and (4) firing pattern is related to firing rate, probably more closely than to cell type.

NON-CHEMICAL-SYNAPTIC MECHANISMS OF SYNCHRONIZATION OF SCN NEURONS

Several independent observations concerning the SCN and circadian rhythms have suggested that SCN neurons can be synchronized by mechanisms that do not involve Na^+-mediated action potentials and chemical synaptic transmission (e.g., Schwartz et al., 1987). When hypothalamic slices were bathed in a $[Ca^{2+}]$-free solution for several hours, bursts of action potentials occurred in the SCN (Bouskila and Dudek, 1993). Multiple-unit recordings showed that populations of SCN neurons had their bursts of activity roughly synchronized, and dual recordings from adjacent areas confirmed that in one SCN the bursts occurred synchronously across the population. The bursts in one SCN were not, however, synchronized with bursts in the contralateral SCN. A mixture of NMDA, non-NMDA, and $GABA_A$ receptor antagonists had no effect upon the synchronicity of the bursts. Whole-cell patch clamp recordings confirmed that the $[Ca^{2+}]$-free solution blocked the evoked EPSPs, and that the mixture of

antagonists blocked the remaining spontaneous postsynaptic potentials. These results indicate that synchronous neuronal activity can occur in the SCN without active chemical synapses, thus strongly suggesting that a different mechanism of communication exists in the SCN.

DISCUSSION AND CONCLUSIONS

Previous extracellular studies provided evidence that retinal input via the optic nerve had an excitatory effect on SCN neurons, and that glutamate acting on non-NMDA receptors was the probable transmitter (Cahill and Menaker, 1989a,b). The preliminary studies of Thomson and West (1990) suggested that GABA was the transmitter responsible for spontaneous IPSPs in SCN neurons. We found that retinal input generated fast EPSPs, but no IPSPs; stimulation of other sites around the SCN caused both fast EPSPs and fast IPSPs (Kim and Dudek, 1991, 1992). Glutamate, acting on non-NMDA receptors at resting potential and on NMDA receptors when the neurons are depolarized, appears to mediate most if not all fast EPSPs (Kim and Dudek, 1991). Only fast IPSPs (no slow IPSPs) were observed in SCN neurons; $GABA_A$ receptors and an increase in Cl^- conductance appeared to generate these IPSPs (Kim and Dudek, 1992). The pharmacological and ionic properties of fast synaptic events appear to be identical to those in other hypothalamic neurons, including those in the supraoptic, paraventricular, and arcuate nuclei (van den Pol et al., 1990; Wuarin and Dudek, 1993).

Although intrinsic electrophysiological properties were described in previous intracellular studies of the SCN (see the introduction), few or no data are available on whether the electrical properties are the same across all the neurons in the SCN. In our experiments, the electrophysiological properties of SCN neurons did not appear to be homogeneous, since some neurons had low-threshold Ca^{2+} spikes and time-dependent inward rectification and yet others did not (Kim and Dudek, 1993). One classification scheme, based on the electrical activity of SCN neurons, has relied on the type of firing pattern (i.e., regular vs. irregular). Our experiments, however, suggest that firing pattern is a function of membrane potential and firing rate (see Thomson et al., 1984). Therefore, although the electrical properties of SCN neurons do not appear homogeneous, distinct classes of SCN neurons based on electrophysiological characteristics are not yet available. Future whole-cell patch clamp studies may yet identify specific classes of SCN neurons.

The conclusion that the SCN displays a circadian rhythm of electrical activity is derived from studies using populations of single-unit recordings (e.g., Gillette, 1991); therefore, the circadian rhythm of electrical activity appears to be a property of a population of SCN neurons, which strongly suggests that a mechanism exists to synchronize at least some of the SCN neurons involved in circadian timekeeping. Our experiments showed that synchronization of neuronal activity into bursts of action potentials could occur in $[Ca^{2+}]$-free solutions that block chemical synaptic transmission (Bouskila and Dudek, 1993). Therefore, although excitatory and inhibitory amino acids appear to mediate all fast postsynaptic potentials in the SCN, Ca^{2+}-dependent chemical synaptic transmission is not necessary for neuronal synchronization. The possible mechanisms include electrotonic coupling via gap junctions among SCN neurons, ephaptic interactions, and shifts in the concentration of extracellular ions such as K^+. Future experiments will attempt to evaluate these mechanisms in the SCN.

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LOCAL INHIBITORY SYNAPTIC INPUTS TO NEURONES OF THE PARAVENTRICULAR NUCLEUS IN SLICES OF RAT HYPOTHALAMUS

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SUMMARY

1. Intracellular recordings were obtained from neurones in the region of the paraventricular nucleus in slices of rat hypothalamus. Glutamate microdrops were applied to the surface of the slices at sites dorsal, lateral and ventral to the paraventricular nucleus to selectively activate local presynaptic neurones. The γ-aminobutyric acid$_A$ ($GABA_A$)-receptor antagonists picrotoxin or bicuculline were bath-applied to block synaptic inhibition.

2. Glutamate microapplication caused a tonic depolarization and often repetitive action potentials in twenty of forty-seven recorded cells. This was probably caused by the direct exposure of the dendrites of the recorded cells to the glutamate microdrops.

3. Glutamate microstimulation elicited inhibitory synaptic responses in nine of forty-seven neurones tested. Glutamate microdrops caused discrete, hyperpolarizing postsynaptic potentials (PSPs) in four cells recorded with microelectrodes containing potassium acetate and evoked depolarizing PSPs in four cells recorded with KCl-filled microelectrodes. Glutamate microapplication inhibited spontaneous spike firing in another cell recorded with a potassium acetate microelectrode.

4. Bath application of $GABA_A$-receptor antagonists completely blocked the hyperpolarizing PSPs elicited by glutamate microstimulation in three of three cells recorded with potassium acetate electrodes and the depolarizing PSPs in two of two cells recorded with KCl electrodes, indicating they were inhibitory PSPs caused by the release of GABA. Suppression of $GABA_A$-mediated synaptic inhibition did not reveal any glutamate-evoked excitatory PSPs.

5. Recorded cells were identified as magnocellular, parvocellular or non-paraventricular bursting neurones on the basis of their electrophysiological properties. Direct depolarization and local inhibitory synaptic responses were observed in all three cell types.

6. Several conclusions can be drawn from these data: (1) functional glutamate

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receptors are distributed throughout neuronal populations in the paraventricular region of the hypothalamus, confirming and extending previous observations; (2) local synaptic inputs to neurones in the paraventricular nucleus are primarily inhibitory, supplied by perinuclear GABAergic neurones; (3) both magnocellular and parvocellular subpopulations receive local inhibitory synaptic inputs. The possibility that these local GABAergic circuits mediate inhibitory inputs to paraventricular neurones from limbic structures is discussed.

INTRODUCTION

The paraventricular nucleus of the hypothalamus is a complex structure comprised of several different populations of cells divided into two main groups, the magnocellular and the parvocellular neurones. The magnocellular neurones are neuroendocrine cells which, together with the magnocellular neurones of the supraoptic nucleus, project to the neurohypophysis and secrete oxytocin or vasopressin. The parvocellular neurones consist of two distinct cell populations, the neurosecretory neurones and the preautonomic neurones. The parvocellular neurosecretory cells project to the median eminence and release hypophysiotrophic factors, which regulate hormone secretion from the anterior pituitary. The preautonomic neurones are not neurosecretory, but project caudally to autonomic centres in the brainstem and spinal cord (Armstrong, Warach, Hatton & McNeill, 1980; Swanson & Kuypers, 1980; Sawchenko & Swanson, 1982).

The magnocellular and parvocellular neurones of the paraventricular nucleus have been found to have different electrophysiological properties such that often they can be identified from intracellular recordings without the need to perform subsequent anatomical analyses (Tasker & Dudek, 1991; Hoffman, Tasker & Dudek, 1991). Magnocellular neurones of the paraventricular nucleus have electrical properties very similar to magnocellular neurones of the supraoptic nucleus, including evidence of a prominent A current and linear current–voltage relations; parvocellular neurones as a group differ from magnocellular neurones by their capacity to generate small low-threshold Ca^{2+} spikes. A third population of neurones with distinct electrical properties has been identified just outside the paraventricular nucleus. These cells generate large low-threshold Ca^{2+} spikes and bursts of action potentials and sometimes display repetitive bursting behaviour (Poulain & Carette, 1987; Tasker & Dudek, 1991). Their close proximity to paraventricular magnocellular neurones and the observation that they sometimes project into the lateral magnocellular region of the paraventricular nucleus has led to the speculation that these bursting cells may provide local synaptic inputs to paraventricular neurones (Poulain & Carette, 1987).

The evidence from several areas of the nervous system (e.g. the retina, the ventral horn of the spinal cord and the CA3 region of the hippocampus) suggests that local synaptic circuits are an important element of the synaptic organization governing neuronal output (see Shepherd, 1990). Evidence for local synaptic regulation of hypothalamic neurones has come primarily from anatomical and indirect physiological studies. According to these reports, the paraventricular nucleus receives projections from several different hypothalamic areas, including the preoptic area, the ventromedial nucleus, the anterior hypothalamus, the lateral hypothalamus, the

suprachiasmatic nucleus and the arcuate nucleus (see Swanson & Sawchenko, 1983). Connections between the bilateral supraoptic and paraventricular nuclei are suggested by the observation that oxytocin or acetylcholine applied to cells of one nucleus *in vivo* facilitates the firing of oxytocinergic neurones in the contralateral nucleus (Moos & Richard, 1989). The electron microscopic finding that oxytocin-containing synaptic terminals contact synaptically with oxytocinergic neurones in the supraoptic nucleus provides anatomic support for these connections (Theodosis, 1985). Inhibitory postsynaptic potentials (IPSPs) evoked in supraoptic magnocellular neurones by electrical stimulation of the diagonal band of Broca have been attributed to local inhibitory interneurones (Randle, Bourque & Renaud, 1986), but the possibility that they were mediated by projections from extrinsic neurones cannot be excluded. Thus data are available which suggest the existence of local synaptic inputs to hypothalamic neurones, but local synaptic circuits have yet to be demonstrated conclusively.

Electrical stimulation is the most commonly used method for studying synaptic circuitry. This technique evokes action potentials in local cell bodies and dendrites, as well as in axons projecting into the region of stimulation from extrinsic sources. It therefore cannot be used to selectively study synaptic interactions between specific populations of neurones without potential contamination from axons of passage. The techniques of simultaneous intracellular and extracellular or paired intracellular recordings from two cells are the most definitive means of determining synaptic connectivity. However, these are technically the most problematic procedures due to the difficulty of obtaining two recordings simultaneously and the need to record from large numbers of neurones in order to overcome the inherent sampling bias. Furthermore, dual recordings are the least likely to provide useful data when local synaptic connections are sparse, since negative data in this case have limited value. Both with extracellular recordings *in vivo* (Goodchild, Dampney & Bandler, 1982) and with intracellular recordings *in vitro* (Christian & Dudek, 1988), glutamate has been shown to evoke action potentials only when applied to neuronal cell bodies and dendrites, and not when applied to axons of passage. Glutamate microapplication, therefore, provides a means by which selective stimulation of specific populations of local neurones can be achieved. By recording postsynaptic events in a neurone in response to focal glutamate microstimulation, one can determine whether the recorded cell receives synaptic inputs from neurones located in the region of glutamate application. This technique effectively reduces the sampling bias encountered with dual recordings, since glutamate microdrops activate groups of cells rather than single neurones.

We have performed experiments using intracellular recordings and glutamate-microdrop application in hypothalamic slices to study local synaptic circuits in the paraventricular nucleus. We have found physiological evidence for glutamate receptors and for local inhibitory synaptic circuits among neurones in the region of the paraventricular nucleus. A preliminary report of these findings has appeared (Tasker & Dudek, 1988).

METHODS

Slice preparation

Adult Sprague–Dawley rats (150–250 g, $n = 41$) were decapitated and their brains removed and immersed in cold (1–2 °C), oxygenated artificial cerebrospinal fluid (ACSF) for 1 min. One or two coronal hypothalamic slices, 400–500 μm in thickness, were cut just caudal to the optic chiasm with a vibroslice tissue slicer (Campden Instruments) and placed in a ramp-style interface recording chamber. The ACSF contained (mM): 124 NaCl, 3 KCl, 2·4 $CaCl_2$, 26 $NaHCO_3$, 1·3 $MgSO_4$, 1·24 NaH_2PO_4 and 11 glucose. The concentrations of $CaCl_2$ and of $MgSO_4$ were raised to 4 mM in some experiments in which KCl-filled recording electrodes were used in order to reduce spontaneous synaptic activity. ACSF was heated to 32–34 °C and pumped into the recording chamber where it was drawn up over the slices with threads of gauze. A gas mixture of 95% O_2 and 5% CO_2 was humidified and directed over the surface of the slices. Slices were allowed to equilibrate in the recording chamber for approximately 2 h prior to the start of experiments.

Electrophysiological techniques

Recording electrodes were pulled from glass capillaries (1·0 mm o.d., 0·5 mm i.d., American Glass Co.) on a Flaming-Brown puller (Sutter Instruments) and filled with 4 M potassium acetate or 2 M KCl. Electrode resistances ranged from 75 to 150 MΩ. Cell impalement was achieved by advancing electrodes through the slice in 4 μm steps with a piezoelectric microdrive (Nanostepper, Adams List) and oscillating the negative capacitance feedback. Electrical signals were recorded using an intracellular amplifier with a bridge circuit (Neurodata Instruments or Axon Instruments), stored on videotape and plotted on a laser printer or played out directly on a pen recorder.

Glutamate microapplication

Glutamate microapplication was used to stimulate selectively the somata and dendrites of local hypothalamic neurones without activating axons projecting to the paraventricular nucleus from extrinsic sources. The purpose of glutamate microapplication, therefore, was to evoke a synaptic response in recorded cells by directly activating presynaptic neurones situated just outside the paraventricular nucleus. Glutamate (10–100 mM in ACSF) was micro-applied by pressure (18–36 N) to the surface of the slice with a single- or double-barrelled micropipette (5–10 μm i.d.). Glutamate microdrops measured approximately 100–250 μm in diameter when applied on a flat surface (e.g. on a microscope slide). Cells were tested for synaptic responses to glutamate at depolarized and hyperpolarized membrane potentials to increase the driving force for inhibitory and excitatory synaptic currents, respectively. When cells did not respond to glutamate microapplication or when glutamate microdrops caused a direct depolarization of the recorded cell, the glutamate micropipette was repositioned until a seemingly pure synaptic response was elicited. For each recorded cell, glutamate was first applied at a site lateral to the paraventricular nucleus. The glutamate micropipette was then moved and glutamate reapplied usually at one or more positions ventral and/or dorsal to the paraventricular nucleus. In some experiments, ACSF was applied through the second barrel of the pipette in the same location and with the same pressure pulses as the glutamate to control for mechanical artifacts of microdrop application.

Drug application

Picrotoxin (50 μM) or bicuculline (10 μM) was added to the perfusate to block $GABA_A$-receptor-mediated inhibition. $GABA_A$ antagonists were applied to block glutamate-evoked IPSPs as well as to reveal local excitatory synaptic circuits that might otherwise be undetectable due to presynaptic inhibition or postsynaptic shunting of synaptic currents.

RESULTS

A total of forty-seven neurones in the region of the hypothalamic paraventricular nucleus were recorded intracellularly and tested for local synaptic inputs with glutamate microstimulation. Recorded cells had a mean resting membrane potential of $62·1 \pm 2·1$ mV (S.E.M., $n = 16$), input resistance of 247 ± 15 MΩ ($n = 22$) and action potential amplitude of $61·6 \pm 1·5$ mV, measured from threshold to peak ($n = 22$).

Direct activation by glutamate

In twenty of the cells tested, glutamate microapplication in one or more sites elicited a tonic depolarization which often caused repetitive spike firing and spike inactivation (Fig. 1). Depolarization was presumably caused by diffusion of the

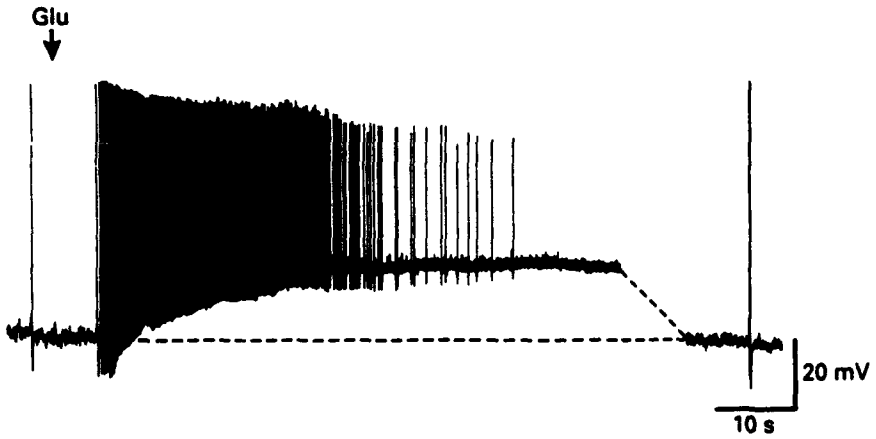

Fig. 1. Direct activation of a paraventricular neurone by glutamate microapplication. Glutamate microdrops (Glu) caused depolarization and repetitive action potentials, suggesting the glutamate was acting directly on the recorded cell. Gradual inactivation of action potential discharge occurred with maintained depolarization. The membrane potential returned to resting level (dashed line) and spontaneous action potentials were seen again after several tens of seconds (break in trace). This cell was a putative parvocellular neurone.

glutamate microdrops such that the glutamate came into direct contact with the dendrites of the recorded cells. In some cases, the recorded cell did not recover from the robust excitation by glutamate. In experiments in which the recorded cell did recover, the glutamate pipette was moved to one or more different positions (see Methods) and the application repeated in order to determine if a synaptic response could be evoked at another site.

Glutamate-evoked synaptic responses

Glutamate microdrops were usually applied at two or more positions around the paraventricular nucleus and, whenever possible, recorded cells were tested for synaptic responses at several different membrane potentials (see Methods). Glutamate microapplication lateral or ventrolateral to the paraventricular nucleus elicited a distinct inhibitory synaptic response in nine of forty-seven cells. In cells recorded with potassium acetate electrodes, glutamate microdrops caused repetitive, hyperpolarizing postsynaptic potentials (PSPs) in four cells (Fig. 2). The latency-to-onset of the PSPs was 0·1 to 3·2 s and the duration was 1·4 to 42 s; the duration of the train of PSPs varied with the size of the glutamate drop, larger drops eliciting responses of longer duration. Bath application of picrotoxin (50 μM) or bicuculline (10 μM) blocked the hyperpolarizing PSPs in three of three neurones tested (Fig. 3),

indicating that they were IPSPs generated by the release of GABA and mediated by $GABA_A$ receptors. The glutamate-evoked IPSPs summated to cause a tonic hyperpolarization in one of these cells (Fig. 4*A*): this cell was one of the three in which the IPSPs were blocked by bicuculline (Fig. 4*B*). Glutamate microapplication

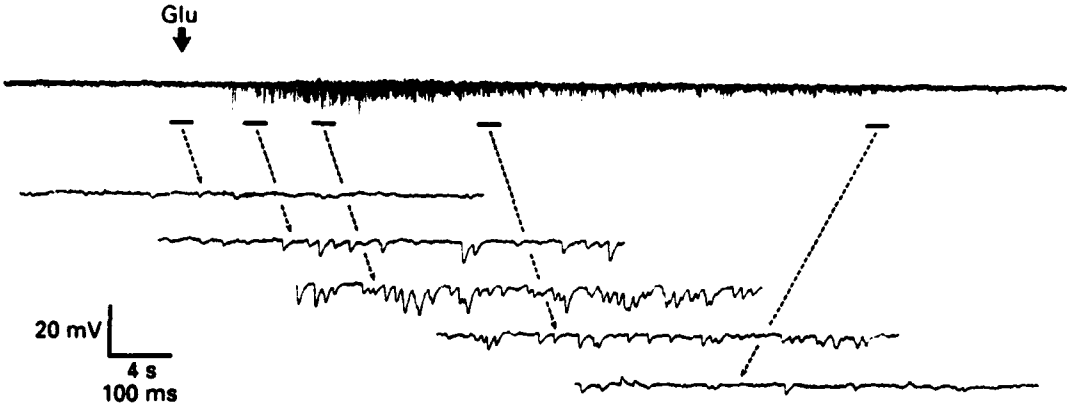

Fig. 2. IPSPs elicited in a cell in the paraventricular nucleus by glutamate microstimulation of local presynaptic neurones. The cell was depolarized approximately 10 mV with continuous positive current to increase the driving force of the Cl^- current; very little spontaneous synaptic activity was seen. Glutamate microdrops applied ventrolaterally to the paraventricular nucleus (Glu) elicited a robust burst of IPSPs which lasted for over 40 s (top trace). Segments of the top trace (bars) were expanded to show individual IPSPs (lower traces). Glutamate microapplication at sites lateral and dorsal to the paraventricular nucleus were less effective or ineffective, respectively, in eliciting a synaptic response. This cell showed electrical properties that were characteristic of magnocellular neurones, including phasic firing with tonic suprathreshold depolarization, and was located within the paraventricular nucleus. The upper time calibration pertains to the top trace and the lower calibration to the expanded traces.

in a fifth cell recorded with a potassium acetate electrode inhibited spontaneous spike firing. Of nine cells recorded with KCl electrodes, four responded to glutamate microdrops with repetitive, depolarizing PSPs. Bicuculline (10 μM) blocked the depolarizing PSPs in two of two cells tested, confirming that they were reversed IPSPs.

Clear EPSPs without tonic depolarization were not observed in response to glutamate microstimulation. Glutamate microdrops did cause an increase in EPSPs in three cells, but the EPSPs were accompanied or preceded by a depolarization. In one of these cells, summated EPSPs appeared to cause the membrane depolarization, but it was not possible to show conclusively that glutamate was not also acting directly on the cell membrane to cause depolarization. In a fourth cell, glutamate microdrops elicited an increase in PSPs, but it was not clear whether these events were EPSPs or IPSPs due to vigorous spontaneous synaptic activity. We were unable to determine whether the glutamate-evoked excitatory responses were purely synaptic in nature (and not also direct) and thus we cannot exclude the possibility that the glutamate microdrops were acting on the presynaptic terminals of projection neurones and not on the somata and dendrites of local presynaptic

neurones. Finally, blockade of synaptic inhibition with picrotoxin or bicuculline failed to reveal EPSPs in response to glutamate microstimulation in a total of seven cells tested.

Cell identification

Recorded cells were identified on the basis of electrophysiological criteria defined in previous reports (Tasker & Dudek, 1991; Hoffman *et al.* 1991). As described in

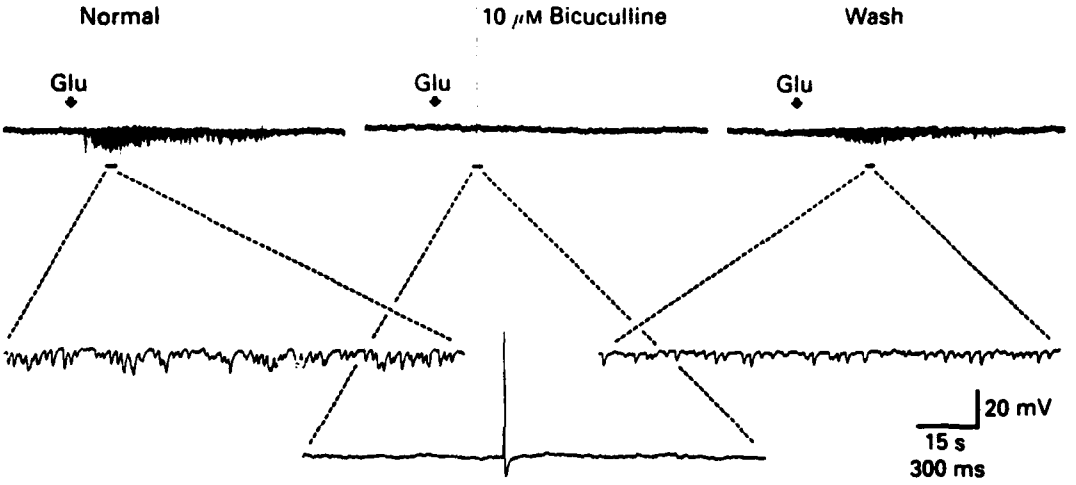

Fig. 3. Glutamate-evoked IPSPs were blocked by $GABA_A$-receptor antagonists. This recording is from the same putative magnocellular neurone as the recording shown in Fig. 2. The cell was depolarized approximately 10 mV with continuous current such that the membrane potential was just subthreshold for action-potential generation. The lower traces are expanded sweeps of the segments of the upper traces designated by the bars. Left traces, a glutamate microdrop applied in normal medium (Glu) evoked a burst of IPSPs. Middle traces, bath application of bicuculline (10 μM) blocked the synaptic response to an identical glutamate microdrop. The drop was applied in the same location and with the same application parameters as the microdrop applied in normal solution (i.e. the pipette was not moved and the pressure settings were not altered). There was no evidence for glutamate activation of local excitatory inputs, since EPSPs were not detected when inhibition was blocked. An action potential was generated from a single EPSP, which probably occurred spontaneously. Right traces, partial recovery of the inhibitory synaptic response to glutamate microstimulation was recorded after 1 h of wash-out of the bicuculline solution. The upper time calibration applies to the top traces and the lower calibration to the expanded traces.

those studies, the main electrical criterion used to identify the different cell populations was the capacity to generate low-threshold Ca^{2+} spikes and the amplitude of the low-threshold Ca^{2+} spikes (i.e. the capacity of the low-threshold spikes to generate bursts of fast action potentials); other distinguishing properties were current–voltage relations, evidence of a distinct A current and/or spontaneous bursting patterns (e.g. phasic firing). Thus, putative magnocellular neurones lacked low-threshold Ca^{2+} spikes (LTS) and showed evidence of a prominent A current (non-LTS cells). Putative parvocellular neurones generated small low-threshold Ca^{2+}

spikes which did not elicit bursts of fast action potentials (non-bursting LTS cells). Non-paraventricular bursting neurones generated large low-threshold Ca^{2+} spikes supporting bursts of fast action potentials and showed strong inward rectification (bursting LTS cells).

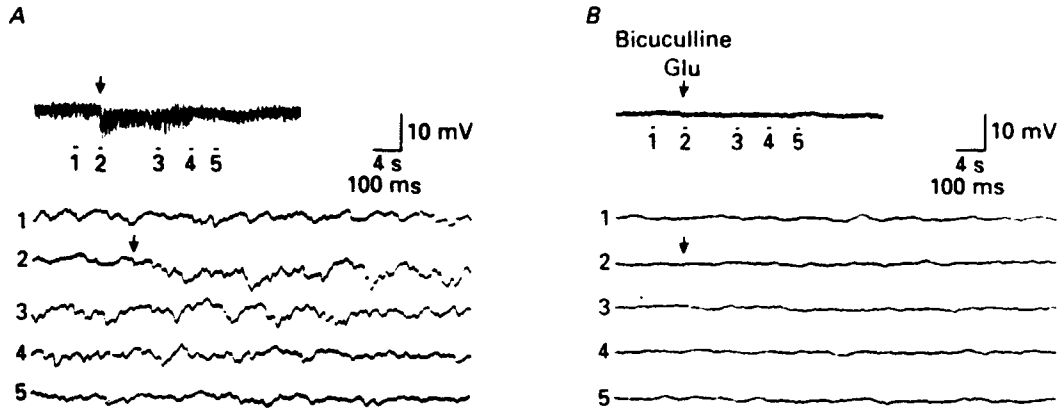

Fig. 4. Hyperpolarization evoked by glutamate microstimulation. This cell showed vigorous on-going synaptic activity. The lower traces labelled 1 to 5 in *A* and *B* are expansions of the segments in the upper traces designated by the bars with corresponding numbers. *A*, in normal ACSF, a glutamate microdrop applied lateral to the paraventricular nucleus elicited a hyperpolarization of the membrane potential. The hyperpolarization apparently was caused by the summation of multiple IPSPs, seen more clearly in the expanded traces below. *B*, bath application of bicuculline (10 μM) blocked not only the IPSPs evoked by the glutamate microdrop, but also the spontaneous IPSPs. No obvious glutamate-evoked EPSPs were detected when $GABA_A$-receptor-mediated inhibition was blocked. This cell had the electrical properties of a non-paraventricular bursting cell and was located dorsal to the paraventricular nucleus, suggesting it was neither a magnocellular nor a parvocellular neurone. The upper time calibrations apply to the top traces in *A* and *B* and the lower calibrations to the expanded sweeps below.

Cells of the three cell types were directly activated by glutamate microdrops. Four of the twenty cells depolarized by glutamate microapplication were putative magnocellular neurones. Ten of the cells were putative parvocellular neurones. One cell was identified as a non-paraventricular bursting neurone from its electrical properties. We did not identify five of the cells directly activated by glutamate microdrops.

Glutamate microstimulation did not evoke a synaptic response at all locations around the paraventricular nucleus. Glutamate microdrops were only effective in eliciting a synaptic response when applied lateral or ventrolateral to the nucleus (Fig. 5). Of the nine cells showing an inhibitory synaptic response to glutamate microstimulation, four were identified as putative magnocellular neurones, four as putative parvocellular neurones and one as a non-paraventricular bursting cell (based on their electrical properties). One of the putative magnocellular neurones generated a phasic firing pattern when depolarized with continuous positive current injection, suggesting that it may have been a vasopressinergic cell (Poulain & Wakerley, 1982).

DISCUSSION

Glutamate microstimulation

Intracellular recordings and glutamate microstimulation were used to study local synaptic interactions among neurones in the paraventricular region of the

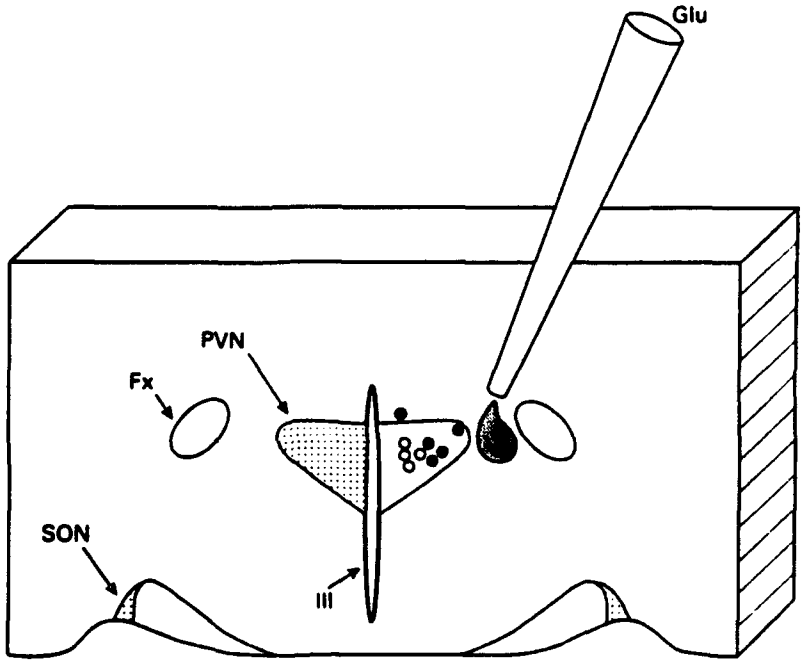

Fig. 5. Neurones responding synaptically to glutamate microstimulation. Glutamate (Glu) microdrops were usually applied in two or more positions around the paraventricular nucleus (PVN); iPSPs were elicited in positions lateral/ventrolateral to the paraventricular nucleus, as indicated by the glutamate drop. Approximate localization of cells responding synaptically to glutamate was achieved from visual placement of the recording electrode with respect to the paraventricular nucleus and landmarks such as the third ventricle (III) and the fornix (Fx). Responsive cells were identified as putative parvocellular (○), magnocellular (●) or non-paraventricular bursting cells (⊕) on the basis of their electrical properties. SON, supraoptic nucleus.

hypothalamus. Glutamate microapplication allowed us to stimulate hypothalamic cells selectively without activating axons of extrinsic neurones (Goodchild *et al.* 1982; Christian & Dudek, 1988). That PSPs in response to glutamate microstimulation were not mediated by receptors located on presynaptic terminals in our experiments (Forsythe & Clements, 1990) is suggested by the pure synaptic response (i.e. without direct depolarization) observed in nine of the recorded cells. Glutamate acting at receptors on presynaptic terminals would be expected to diffuse also to the receptors located on the postsynaptic membrane, which would cause direct depolarization in addition to synaptic activation of the postsynaptic cell. Synaptic activation with glutamate microstimulation was usually specific to the site of microdrop application

(i.e. glutamate microdrops were not effective in eliciting a synaptic response at all application sites around the paraventricular nucleus). Therefore, it should be possible in the future with this technique to map topographically the areas around (or inside) the paraventricular nucleus that contain cells presynaptic to paraventricular neurones.

Direct activation with glutamate

As in other structures of the central nervous system, glutamate is thought to be the main excitatory neurotransmitter in the hypothalamus. Hypothalamic neurones express genes which encode for glutamate receptors (Bettler *et al.* 1990). Iontophoretic application of glutamate activates cells in the supraoptic (Bioulac, Gaffori, Harris & Vincent, 1978; Arnaud, Cirino, Layton & Renaud, 1983) and paraventricular nuclei (Moss, Urban & Cross, 1972). Synaptic responses evoked by electrical stimulation of afferents to neurones in the supraoptic (Gribkoff & Dudek, 1990) and paraventricular nuclei (Wuarin & Dudek, 1991) are blocked by glutamate-receptor antagonists. In our experiments, glutamate microdrops caused tonic depolarization and spike generation in putative magnocellular and parvocellular neurones of the paraventricular nucleus and in non-paraventricular bursting cells. This response was presumably caused by glutamate diffusion into the nucleus (in the case of magnocellular and parvocellular neurones) where it acted directly on the dendrites or cell bodies of paraventricular neurones. These data confirm the previous evidence for glutamate receptors on paraventricular neurones and indicate that both magnocellular and parvocellular neurones, as well as non-paraventricular bursting neurones, have functional glutamate receptors. Our finding that glutamate activates local inhibitory neurones presynaptic to paraventricular neurones suggests that these perinuclear GABA-containing cells also have functional glutamate receptors. Whether these presynaptic inhibitory neurones and the non-paraventricular bursting neurones are the same cells remains to be determined.

Glutamate-evoked synaptic inhibition

Our results provide physiological evidence for inhibitory synaptic inputs to neurones in the region of the paraventricular nucleus from cells located outside the nucleus. Although the glutamate microdrops were applied outside the boundaries of the paraventricular nucleus on the basis of visual cues, we cannot rule out the possibility that the synaptic responses were mediated by local inputs from cells within the lateral paraventricular nucleus (due to diffusion of the glutamate microdrops into the nucleus). However, in a putative magnocellular neurone that was localized with biocytin injection and neurophysin immunohistochemistry on the lateral border of the nucleus in a separate study (see Fig. 2 of Hoffman *et al.* 1991), a pure synaptic response was evoked by glutamate microstimulation lateral to the recorded cell (i.e. probably outside the nucleus). The GABAergic cells that have been identified immunohistochemically within the paraventricular nucleus tend to be located in medial parvocellular regions (Meister, Hökfelt, Geffard & Oertel, 1988; Decavel & van den Pol, 1990) and it is unlikely that the glutamate microdrops diffused that far medially. Thus, projections from neurones located outside the paraventricular nucleus probably mediated these synaptic responses.

The inhibitory synaptic responses evoked by glutamate were blocked by $GABA_A$-receptor antagonists, suggesting they were mediated by GABA release. This local GABAergic projection to paraventricular cells probably originates in the population of GABA-containing cells that has been described in the area surrounding the paraventricular nucleus in immunohistochemical studies (Meister *et al.* 1988; Sakaue, Saito, Taniguchi, Baba & Tanaka, 1988). GABAergic synapses make up half of the total number of synapses impinging on hypothalamic neurones in the arcuate, supraoptic and paraventricular nuclei (Decavel & van den Pol, 1990), and GABAergic synapses account for most of the 'double' synapses (i.e. one presynaptic element contacting two postsynaptic elements in the same plane of section) which form selectively on supraoptic and paraventricular oxytocinergic cells during lactation (Theodosis, Paut & Tappaz, 1986; Theodosis & Poulain, 1989). Intracellular recordings in the hypothalamic explant have revealed robust inhibitory synaptic inputs to neurosecretory neurones of the supraoptic nucleus occurring spontaneously and in response to electrical stimulation of the diagonal band of Broca (Randle *et al.* 1986). Neurones in the paraventricular nucleus appear to receive an equally dense GABAergic innervation (Mugnaini & Oertel, 1985; Meister *et al.* 1988; Sakaue *et al.* 1988; Decavel, Dubourg, Leon-Henri, Geffard & Calas, 1989; Decavel & van den Pol, 1990) and it is likely that at least a proportion of this input derives from local sources. Indeed, recent anatomical findings from combined tract-tracing and immunohistochemical experiments suggest that a large proportion of the GABAergic inputs to the paraventricular nucleus arise locally (Roland, Brown & Sawchenko, 1991). Our physiological data corroborate these anatomical observations and further suggest that local GABAergic inputs to the paraventricular nucleus may be important for the regulation of both magnocellular and parvocellular neurones.

A relatively small percentage of the recorded cells (19%) showed an inhibitory synaptic response to glutamate microstimulation. Although this may indicate a paucity of inhibitory inputs to neurones of the paraventricular nucleus from perinuclear GABAergic neurones, it may also reflect technical limitations of glutamate microstimulation in the slice preparation. It is possible, for example, that many more paraventricular neurones receive local inhibitory inputs but that the presynaptic neurones are not contained within the slice or that the trajectory of their axons leaves the plane of the slice. Thus negative results cannot be interpreted necessarily as a lack of local synaptic inputs. However, from studies done in neocortical slices, it is clear that the density of local synaptic circuits in the hypothalamus is qualitatively much lower than that in the neocortex (Tasker, Peacock & Dudek, 1992).

Glutamate-evoked synaptic excitation

Although pure EPSPs (i.e. without direct membrane depolarization) were not seen in response to glutamate microstimulation, it is possible that local excitatory synaptic circuits are present in this region of the hypothalamus. Since glutamate-evoked depolarizations were accompanied sometimes by an apparent increase in EPSPs, some of these may have been due to the rapid summation of EPSPs or to the combination of a direct effect on the recorded cell and a simultaneous activation of presynaptic excitatory neurones. As discussed above, the failure to detect pure

EPSPs in response to glutamate microstimulation may have been caused by cutting the local excitatory projections in the slicing procedure (because of their trajectory out of the plane of the slice) or by the glutamate microdrops not reaching the excitatory presynaptic neurones when present in the slice. Nevertheless, the lack of a clear excitatory synaptic response to glutamate microapplication, both in normal solution and with inhibition blocked with GABA antagonists, suggests that local excitatory synaptic circuits are not abundant in this region of the hypothalamus.

Possible functional significance

Although it is premature to draw conclusions as to the physiological significance of a local inhibitory projection to paraventricular neurones, it is reasonable to speculate on the possible involvement of such a projection in neurosecretory function. Several limbic structures have been shown to exert an inhibitory influence on the electrical activity of supraoptic and paraventricular neurones recorded extracellularly *in vivo* (Pittman, Blume & Renaud, 1981; Ferreyra, Kannan & Koizumi, 1983). Stimulation of the septum inhibits the background firing of oxytocinergic neurones in the supraoptic and paraventricular nuclei and alters the periodicity of reflex milk ejections (Lebrun, Poulain & Theodosis, 1983). Autoradiographic and retrograde labelling studies have shown that projections from limbic structures such as the septum and subiculum do not enter the paraventricular nucleus but terminate in the area immediately surrounding it, forming a 'halo' around the nucleus (Sawchenko & Swanson, 1983; Silverman & Oldfield, 1984). The dendrites of paraventricular neurones tend to be restricted to the nucleus, with few dendritic branches extending outside the boundaries of the nucleus (van den Pol, 1982; but see Oldfield, Hou-Yu & Silverman, 1985). These results together suggest that the inhibitory influence limbic structures exercise on neurosecretory systems is transmitted via interneurones located around the paraventricular nucleus. Our findings indicate that there are local GABAergic neurones outside the paraventricular nucleus that provide inhibitory synaptic inputs to both magnocellular and parvocellular neurones, and suggest that the inhibitory influence of limbic structures on paraventricular neurosecretory neurones may be mediated by these perinuclear GABAergic interneurones.

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MEMBRANE PROPERTIES OF RAT SUPRACHIASMATIC NUCLEUS NEURONS RECEIVING OPTIC NERVE INPUT

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SUMMARY

1. The electrophysiological properties of suprachiasmatic nucleus (SCN) neurons ($n = 33$) receiving optic nerve input were studied with intracellular recordings in rat hypothalamic slices maintained *in vitro*. Our major goal was to provide baseline data concerning the intrinsic membrane properties of these neurons and to test the hypothesis that the neurons are homogeneous electrophysiologically.

2. Action potentials were short in duration and followed by a pronounced hyperpolarizing after-potential. Spike amplitude ($58{\cdot}2 \pm 1{\cdot}1$ mV, mean ± S.E.M.; measured from threshold), spike duration ($0{\cdot}83 \pm 0{\cdot}03$ ms; measured at half amplitude) and hyperpolarizing after-potential amplitude ($23{\cdot}9 \pm 1{\cdot}0$ mV; measured from threshold) appeared unimodally distributed and did not co-vary.

3. Intracellular injection of depolarizing current pulses evoked spike trains, and spike inactivation, spike broadening and frequency accommodation were always present. An after-hyperpolarization followed the spike train in all but one neuron.

4. Membrane time constant ranged from 7·5 to 21·1 ms ($11{\cdot}4 \pm 0{\cdot}7$ ms, $n = 27$), and its distribution appeared to be unimodal with the peak at ~ 10 ms. Input resistance ranged from 105 to 626 MΩ (301 ± 23 MΩ, $n = 33$); the distribution also appeared unimodal with its peak at ~ 250 MΩ.

5. A subpopulation (16 of 33, 48%) of the neurons exhibited slight (6–29%) time-dependent inward rectification in their voltage responses to hyperpolarizing current injection. Of the neurons lacking the time-dependent rectification, some ($n = 5$) exhibited time-independent inward rectification of 6–20% and others showed no (or < 3%) such rectification. The degree of inward rectification was correlated with neuronal excitability ($r = 0{\cdot}60$, $P < 0{\cdot}002$; assessed by measuring the steepness of the primary slope of the frequency–current plot) and with the spontaneous firing rate ($r = 0{\cdot}49$, $P < 0{\cdot}007$). Furthermore, the neurons with > 6% inward rectification (neurons with time-dependent and -independent rectification were combined) were more excitable (362 ± 43 Hz/nA ($n = 15$) *vs.* 221 ± 37 Hz/nA ($n = 9$), $P < 0{\cdot}05$) and had a higher spontaneous firing rate ($11{\cdot}1 \pm 1{\cdot}9$ Hz ($n = 19$) *vs.* $3{\cdot}9 \pm 1{\cdot}5$ Hz ($n = 11$), $P < 0{\cdot}02$) than the neurons with no or negligible (i.e. < 3%) inward rectification. The

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two groups, however, were not significantly different in membrane time constant and input resistance.

6. When adequately hyperpolarized, twelve of seventeen (71%) neurons generated small low-threshold spike (LTS) potentials in response to depolarizing current pulses. The neurons with LTS potentials were not significantly different from other neurons ($n = 5$, 29%) in spontaneous firing rate, excitability, membrane time constant and input resistance. The capacity to generate LTS potentials was not related to the presence of inward rectification.

7. Spontaneous firing (up to 34 Hz) was present in all but six neurons. In general, neurons with a firing rate > 6 Hz (71%) had a regular firing pattern, whereas neurons that fired at < 4 Hz (25%) had an irregular firing pattern. Altering the firing rate with intracellular current injection changed the firing pattern. Therefore, depending on the firing rate, a given neuron could show either a regular or an irregular firing pattern.

8. These results suggest that the retinorecipient neurons in the SCN do not form a completely homogeneous group nor clearly distinct classes in terms of intrinsic membrane properties. Furthermore, the data suggest inward rectification may be related to enhanced excitability and firing rate.

INTRODUCTION

Several lines of evidence suggest that, in mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus contains the biological clock responsible for circadian rhythms (for review, see Takahashi & Zatz, 1982; Moore, 1983; Turek, 1985; Meijer & Rietveld, 1989). Entrainment of these rhythms to the daily light–dark cycle is an important process that allows maximal adaptation of an organism to its external environment. In mammals, the photic entrainment is mediated exclusively through the photoreceptors in the retina (Richter, 1965; Nelson & Zucker, 1981). The photic information delivered to the SCN via the retinohypothalamic tract, a direct projection from the retina to the SCN (Hendrickson, Wagoner & Cowan, 1972; Moore & Lenn, 1972), is apparently sufficient, if not essential, to maintain entrainment of circadian rhythms (Rusak & Boulos, 1981; Moore, 1983; Albers, Liou, Ferris, Stopa & Zoeller, 1991).

Realization of the potentially critical role of the retinohypothalamic pathway in photic entrainment has stimulated research concerning the SCN neurons that receive direct retinal input. Immunohistochemical evidence indicates that a subpopulation of these neurons contain vasoactive intestinal polypeptide (Ibata *et al.* 1989), and electrophysiological results strongly suggest that excitatory amino acid receptors mediate the fast retinal input to these neurons (Shibata, Liou & Ueki, 1986; Cahill & Menaker, 1987, 1989*b*; Kim & Dudek, 1991*a*). Limited information is available concerning intrinsic membrane properties of retinorecipient SCN neurons. Although Wheal & Thomson (1984) described several intrinsic membrane properties of SCN neurons, including the current–voltage relation, they did not determine whether the neurons received synaptic input from the optic nerve. They also did not evaluate quantitatively whether the neurons were homogeneous electrophysiologically. More recently, Sugimori, Shibata & Oomura (1986) reported that SCN neurons receiving synaptic input from the optic nerve expressed low-threshold Ca^{2+} spike potentials

and time-dependent inward rectification. However, they did not report how many cells were recorded for their study and what proportion of the cells had these intrinsic properties. Other studies with extracellular recordings from single SCN neurons have reported differences in firing pattern across the retinorecipient cells (Shibata, Oomura, Hattori & Kita, 1984; Cahill & Menaker, 1989*a*), which suggests that these neurons have heterogeneous intrinsic membrane properties.

In the present study, we aimed to provide baseline data concerning the intrinsic membrane properties of retinorecipient cells in rat SCN and to test the hypothesis that the neurons form an electrophysiologically homogeneous population. We also sought to determine if any intrinsic membrane properties were related to neuronal excitability or spontaneous firing rate. The data from these intracellular electrophysiological experiments suggest that the neurons are relatively homogeneous in many intrinsic membrane properties; however, they are also heterogeneous in some properties, such as inward rectification, which might be related to neuronal firing. A preliminary account of these results has been published (Kim & Dudek, 1991*b*).

METHODS

Animals

Male Sprague–Dawley rats ($n = 23$; 120–350 g) purchased from Charles River company (USA) were used for this study. Prior to electrophysiological experiments, the rats were housed in a temperature-controlled room (22–23 °C) under a 12 h light–12 h dark cycle (light on at 07.00 h; pacific standard time) for at least 1 week (mostly > 2 weeks).

Hypothalamic slices

The methods employed for the preparation and maintenance of the slices were the same as those described earlier (Kim & Dudek, 1991*a*). In brief, a rat was decapitated with a guillotine under sodium pentobarbitone (100 mg/kg I.P.) anaesthesia induced after 07.00 h (i.e. after light on), and the brain was removed and submerged in ice-cold physiological saline (composition (mM): 124 NaCl, 1·4 NaH_2PO_4, 3 KCl, 2·4 $CaCl_2$, 26 $NaHCO_3$, 1·3 $MgSO_4$, 11 glucose). After about 1 min chilling, the brain was trimmed to a block containing the entire hypothalamus and optic nerves. Two 500 μm thick parasagittal slices containing the optic nerve and SCN were cut from the block with a vibrating microtome (Campden Instruments, UK). The slices were quickly transferred to an interface-type recording chamber and perfused with physiological saline (32–35 °C) at 0·7–1·0 ml/min. A humidified mixture of 95% O_2 and 5% CO_2 was blown over the slice for the entire experimental period. Electrophysiological recordings started 2 h after the slice preparation.

Intracellular recordings and electrical stimulation

Intracellular recordings were obtained with micropipettes filled with 2–4 M potassium acetate (90–360 MΩ). Voltage recordings in current-clamp mode were performed with a high-impedance amplifier having a 'bridge' circuit (Axoclamp-2A, Axon Instruments, USA). A bipolar metal electrode (90% platinum–10% iridium) was placed in the cut end of the optic nerve for stimulation ($\leq$ 0·8 mA, 0·5 ms monophasic pulse).

RESULTS

The results presented in this paper were from thirty-three SCN neurons that responded to optic nerve stimulation with an excitatory postsynaptic potential (Kim & Dudek, 1991*a*). Stable recordings for > 10 min (up to 6 h) were obtained between 11.00 h and 21.00 h. None of the intrinsic membrane properties examined in this study appeared to be a function of the time of recording.

Action potentials

The shape and characteristics of action potentials were similar between neurons. Figure 1*A* illustrates a typical action potential. The spike amplitude (measured from threshold) was in the range of 50–65 mV (58·2 ± 1·1 mV, mean ± S.E.M.), and the spike

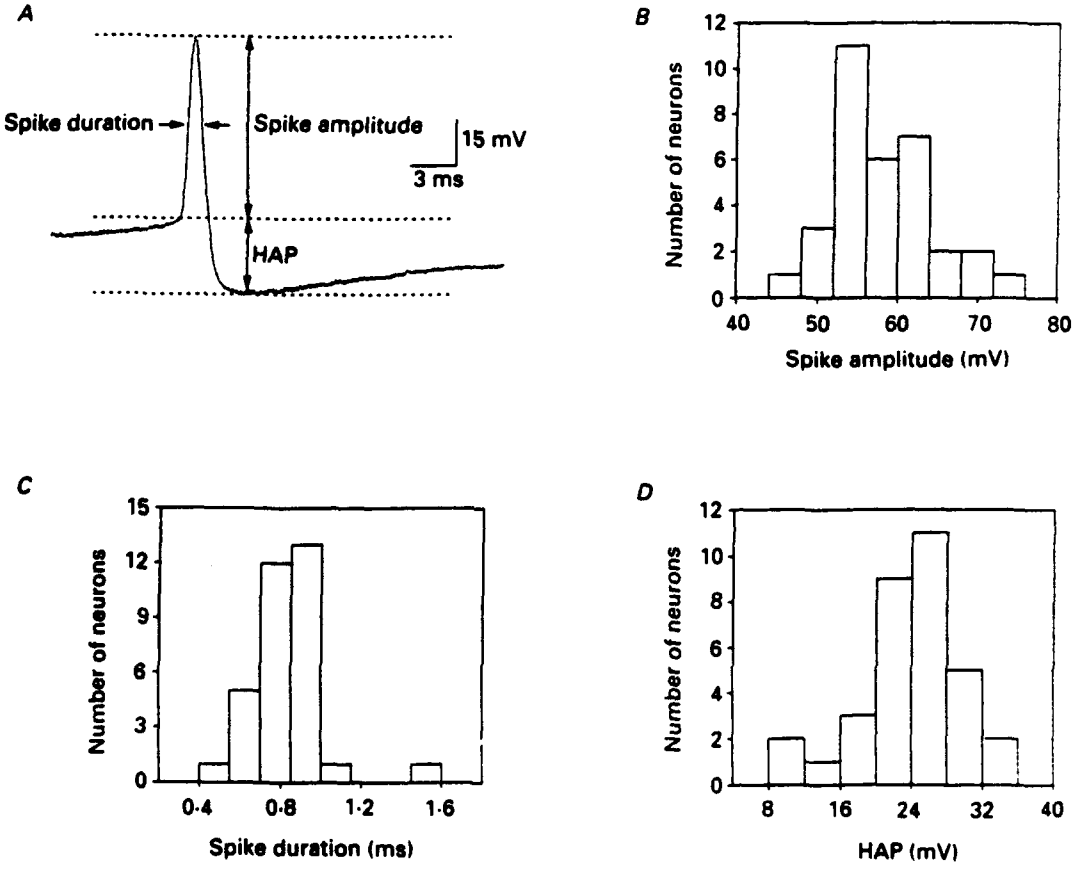

Fig. 1. Characteristics of action potentials. *A*, a representative action potential recorded from an SCN neuron. *B*, *C* and *D*, frequency histograms (33 neurons) for spike amplitude (*B*), spike duration (*C*) and hyperpolarizing after-potential (HAP; *D*).

duration (measured at half amplitude) was relatively short (0·83 ± 0·03 ms). A pronounced (normally > 20 mV) hyperpolarizing after-potential (23·9 ± 1·0 mV; measured from threshold) usually followed each spike. Spike amplitude, spike duration and hyperpolarizing after-potential amplitude appeared unimodally distributed (Fig. 1*B*, *C* and *D*) and did not co-vary (data not shown).

Spike train and after-hyperpolarization

In every neuron examined, intracellular injection of depolarizing current pulses of sufficient intensity elicited spike trains. Spike inactivation, spike broadening and frequency accommodation (Fig. 2) were always present, although there was some variability between the neurons. In all but one neuron, an after-hyperpolarization

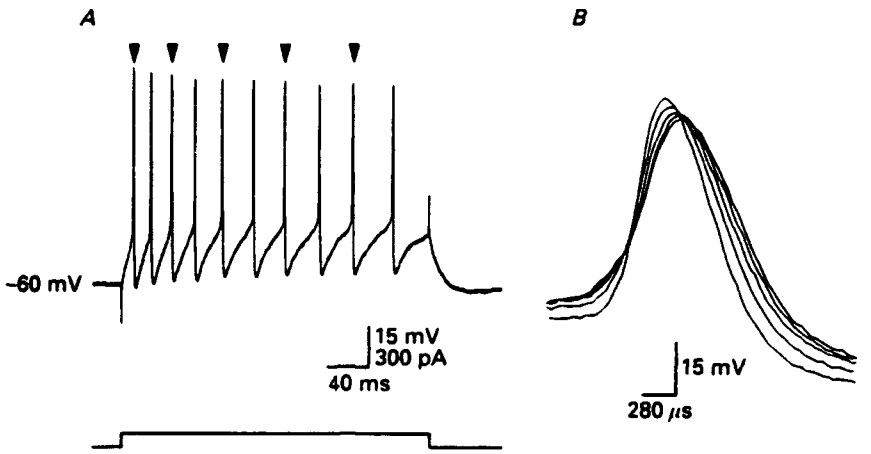

Fig. 2. Properties of the spike train evoked by depolarizing current pulse. *A*, an evoked action potential train exhibiting spike inactivation and frequency accommodation. *B*, the spikes (arrowheads) in *A* are superimposed to illustrate progressive spike broadening and inactivation. Cell was current clamped to −60 mV.

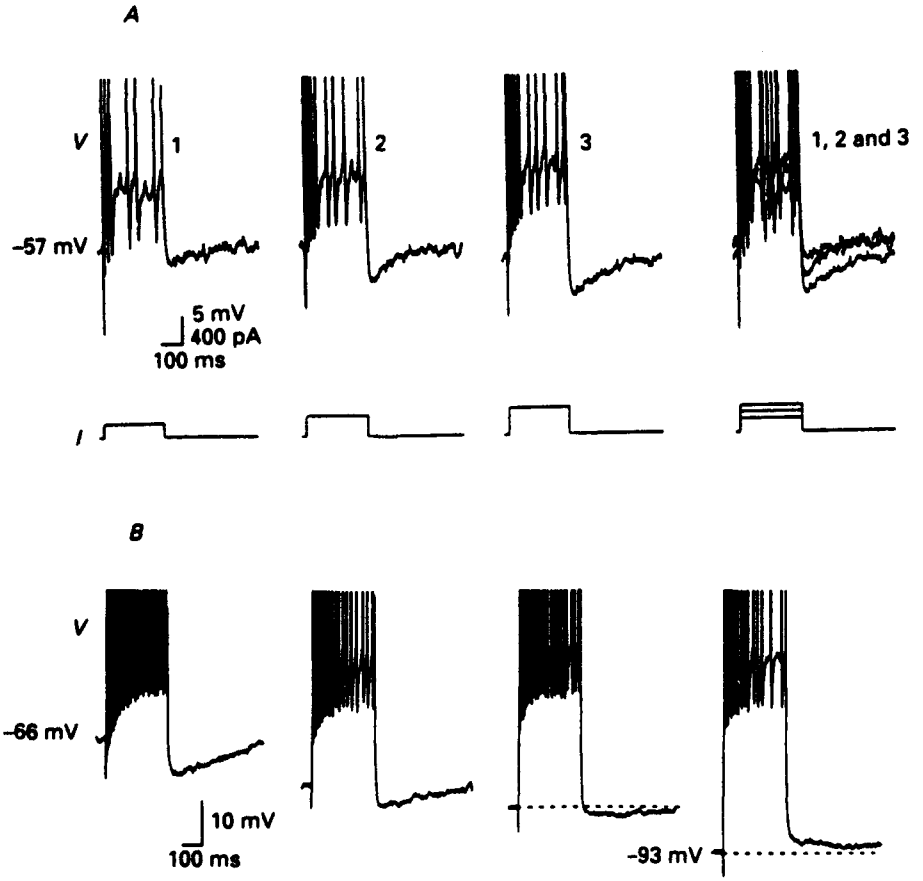

Fig. 3. Properties of the post-train after-hyperpolarization. *A*, spike trains evoked by three different intensities of current pulses. The amplitude of the post-train after-hyperpolarization was a function of current intensity. Cell was current clamped to −57 mV. *B*, in another neuron, spike trains evoked at four different membrane potentials. The polarity of the post-train after-hyperpolarization reversed at around −90 mV.

followed each of the evoked spike trains. The amplitude of the after-hyperpolarization was a function of current intensity (Fig. 3*A*), and its polarity reversed at around −85 to −90 mV (i.e. near the K^+ equilibrium potential) (Fig. 3*B*). The decay of the after-hyperpolarization lasted up to 1 s, and usually could be fitted well with a single exponential.

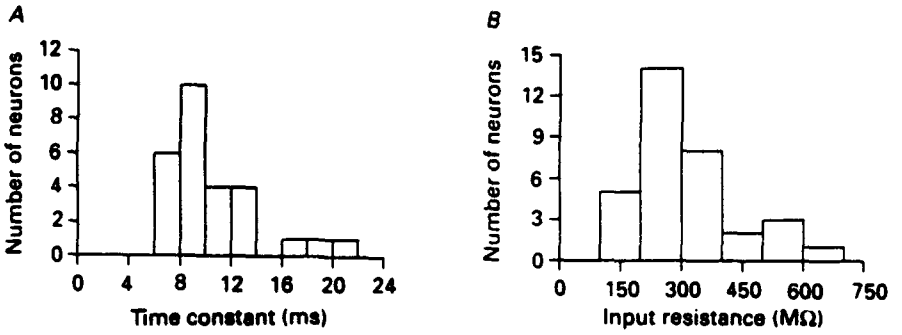

Fig. 4. Frequency histograms for membrane time constant (*A*; $n = 27$ neurons) and input resistance (*B*; $n = 33$ neurons).

Membrane time constant and input resistance

The membrane time constant, which was estimated from the averaged electrotonic potential elicited by a small hyperpolarizing current pulse delivered in the linear portion of the current–voltage (*I–V*) plot, ranged from 7·5 to 21·1 ms (11·4 ± 0·7 ms, $n = 27$). The distribution of the membrane time constant appeared to be unimodal with the peak at ~ 10 ms (Fig. 4*A*).

The input resistance, which was calculated from the slope of the linear portion of the *I–V* plot, was in the range of 105–626 MΩ (301 ± 23 MΩ, $n = 33$). Its distribution (Fig. 4*B*) appeared to be unimodal with the peak at ~ 250 MΩ.

Inward rectification

In sixteen of the thirty-three neurons (48%), time-dependent inward rectification was observed in their voltage responses to hyperpolarizing current injection (Fig. 5). The degree of the time-dependent rectification between the neurons ranged from 6 to 29% (mostly 12–29%). The *I–V* plots for the neurons lacking the time-dependent rectification revealed that five of these neurons had time-independent inward rectification of 6–20%; for other neurons this rectification was < 3% or absent. The degree of rectification was calculated by comparing the maximum and minimum apparent input resistances; the maximum was estimated from a −9 to −22 mV electrotonic potential in the linear portion of the *I–V* plot, while the minimum was from a potential of −36 to −63 mV falling in the most non-linear portion of the *I–V* plot. The electrotonic potentials were evoked from a baseline membrane potential of −56 to −77 mV. The baseline potential was often adjusted with continuous hyperpolarizing current to prevent spontaneous spiking. In silent cells ($n = 6$), the potential was equal to the resting potential.

The degree of inward rectification was correlated with the neuronal excitability ($r = 0·60$, $P < 0·002$; quantified by measuring the steepness of the primary slope of

the frequency–current plot; Fig. 6*A*) and with the spontaneous firing rate ($r = 0{\cdot}49$, $P < 0{\cdot}007$). Furthermore, the neurons with > 6% inward rectification (neurons with time-dependent and -independent rectification were combined), compared to those with no or negligible (i.e. < 3%) inward rectification, were more excitable

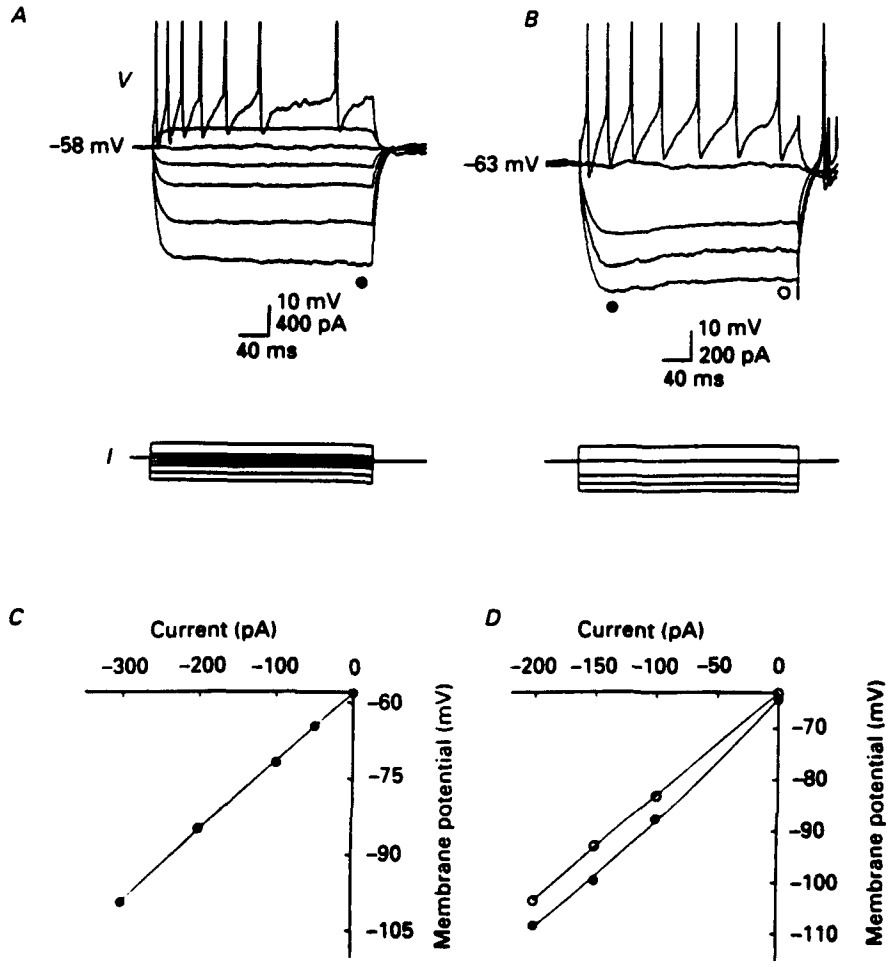

Fig. 5. Time-dependent inward rectification. *A* and *B*, voltage responses (upper traces) of two different neurons to intracellularly injected current pulses (lower traces). Action potentials are truncated. Cells were current clamped to −58 mV (*A*) and −63 mV (*B*). *C* and *D*, *I–V* plots for the data in *A* and *B*, respectively. Voltage measurements were made at the time points indicated with ● and ○. The data in *A* and *C* indicate the lack of inward rectification, whereas those in *B* and *D* illustrate the presence of time-dependent inward rectification.

(362 ± 43 Hz/nA ($n = 15$) *vs.* 221 ± 37 Hz/nA ($n = 9$), $P < 0{\cdot}05$; Fig. 6) and had a higher spontaneous firing rate (11·1 ± 1·9 Hz ($n = 19$) *vs.* 3·9 ± 1·5 Hz ($n = 11$), $P < 0{\cdot}02$; Fig. 7). Between the two groups, however, membrane time constant (11·8 ± 0·9 ms ($n = 15$) *vs.* 10·9 ± 1·0 ms ($n = 12$), $P > 0{\cdot}5$) and input resistance (331 ± 29 MΩ ($n = 21$) *vs.* 248 ± 32 MΩ ($n = 12$), $P > 0{\cdot}05$) were not significantly different.

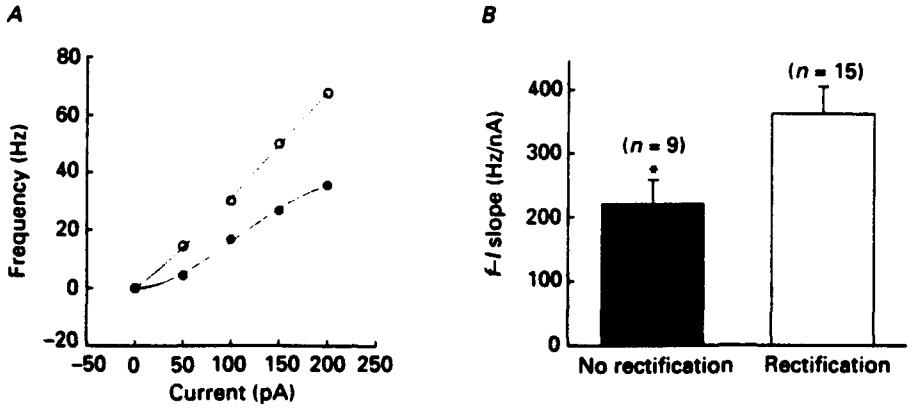

Fig. 6. Neurons with inward rectification were more excitable. *A*, frequency–current (*f–I*) plots for two neurons with (○) and without (●) inward rectification. Cells were current clamped to −69 and −70 mV, respectively. Spike frequency was calculated from the averaged number of spikes evoked by 300 ms current pulses of a given intensity (up to 500 pA). *B*, comparison of the mean (±S.E.M.) primary *f-I* slope (Hz/nA) between neurons with > 6% inward rectification (open bar) and those with no or negligible (i.e. < 3%) rectification (filled bar). $*P < 0.05$.

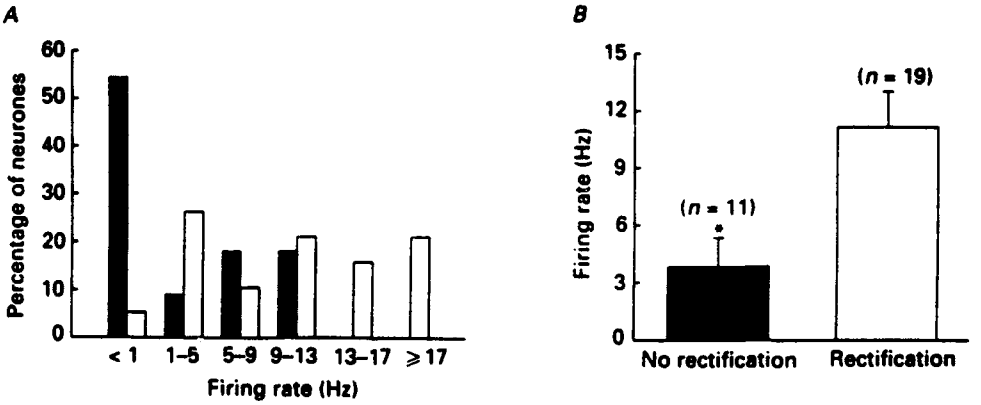

Fig. 7. Neurons with inward rectification had higher spontaneous firing rate. *A*, frequency histogram for firing rate. Note that over half of the cells with no or negligible (i.e. < 3%) inward rectification (filled bars) had a firing rate of < 1 Hz (most were silent) and over half of the cells with > 6% rectification (open bars) had a firing rate of > 9 Hz. *B*, comparison of the mean (±S.E.M.) firing rate between the two neuronal groups. $*P < 0.02$.

Low-threshold spike potentials

Low-threshold spike (LTS) potentials were detected in twelve of seventeen (71%) neurons. These potentials were evoked only when the cell was hyperpolarized adequately (Fig. 8*A*) and only by depolarizing potentials of sufficient amplitude (Fig. 8*B*). The LTS potentials were small; in general, they barely supported single action potentials (Fig. 8*A*), and only rarely two to three action potentials (Fig. 8*B*). Spontaneous firing rate (8·9 ± 2·8 Hz ($n = 12$) *vs.* 9·3 ± 3·3 Hz ($n = 5$), $P > 0.8$), excitability (384 ± 71 Hz/nA ($n = 9$) *vs.* 208 ± 44 Hz/nA ($n = 5$), $P > 0.1$), membrane

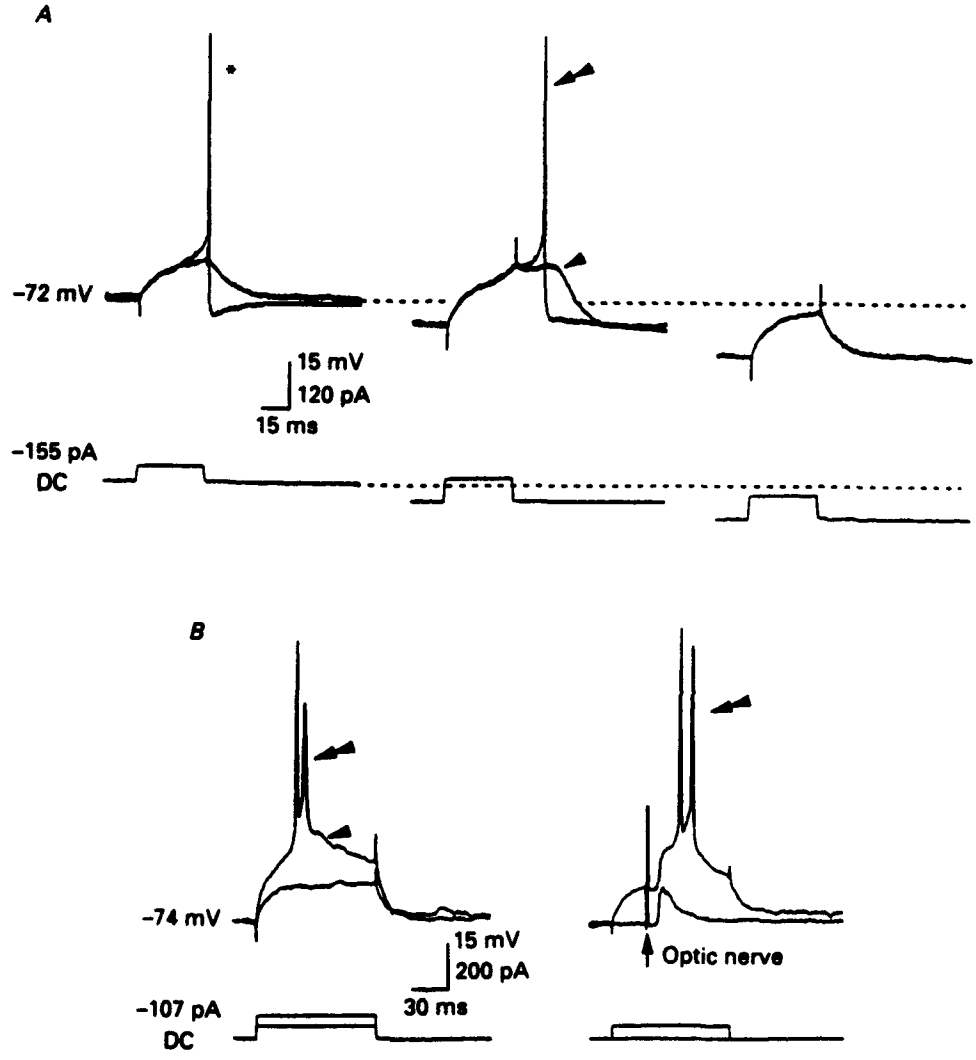

Fig. 8. Characteristics of LTS potentials. *A*, adequate hyperpolarization removed the inactivation of LTS conductance. Left: at a membrane potential of −72 mV, a depolarizing current pulse evoked an electrotonic potential or action potential (*). Middle: when the cell was hyperpolarized to about −80 mV, a depolarizing current pulse could evoke a small LTS potential (arrowhead). Occasionally, an action potential (double arrowheads) arose from the LTS potential. Right: when the cell was hyperpolarized to about −90 mV, a current pulse of the same intensity as the one in the middle traces did not evoke an LTS potential. *B*, depolarizing potentials of only sufficient amplitude evoked LTS potentials. Data from another cell hyperpolarized to −74 mV. Left: of the two different intensities of current pulses injected, the higher one evoked an LTS potential (arrowhead) that supported two action potentials (double arrowheads). Right: when superimposed on a depolarizing electrotonic potential, the excitatory postsynaptic potential (EPSP) from optic nerve stimulation (arrow) evoked an LTS potential similar to the one in the left panel. Two action potentials (double arrowheads) arose from the LTS potential that emerged at the falling phase of the EPSP.

time constant ($12{\cdot}8 \pm 1{\cdot}4$ ms ($n = 11$) *vs.* $9{\cdot}4 \pm 0{\cdot}7$ ms ($n = 5$), $P > 0{\cdot}1$) and input resistance (327 ± 42 MΩ ($n = 12$) *vs.* 240 ± 46 MΩ ($n = 5$), $P > 0{\cdot}2$) were not significantly different between the groups of neurons that did and did not generate the LTS potentials. The capacity of a given neuron to generate an LTS potential was not

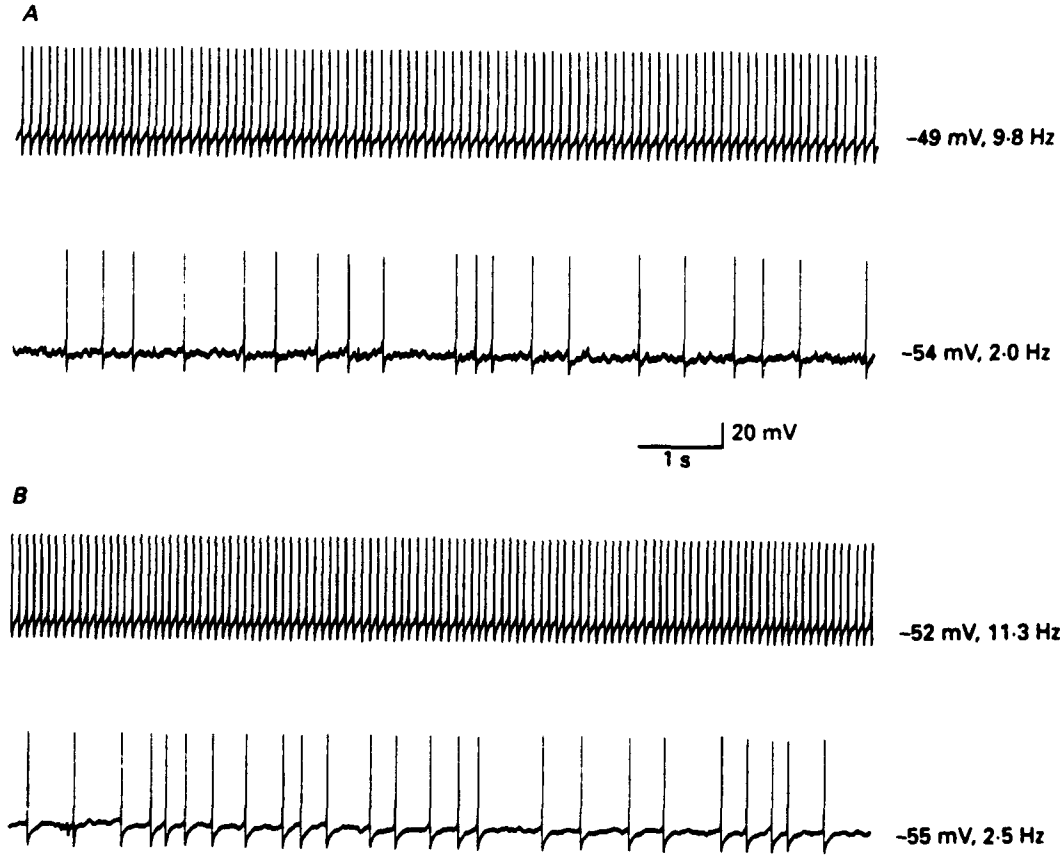

Fig. 9. Firing patterns were related to firing rate. *A*, regular (upper trace) and irregular (lower trace) spontaneous firing patterns observed in two different neurons at rest. *B*, a regular firing pattern observed in a neuron recorded at rest (upper trace), and an irregular pattern recorded from the same neuron at a more negative membrane potential (lower trace). The hyperpolarization was with intracellular injection of cathodal current. In *A* and *B*, the baseline membrane potential and firing rate are indicated to the right of each trace.

related to the presence of inward rectification: seven of twelve neurons that generated LTS potentials had inward rectification, and two of five that did not generate them had inward rectification ($\chi^2 = 0{\cdot}48$, $P > 0{\cdot}25$).

Firing rate and pattern

All of the neurons except six were spontaneously active. In 71% of the cells, the spontaneous firing rate was > 6 Hz (up to 34 Hz), and in 25% it was < 4 Hz. In general, the pattern of > 6 Hz firing was quite regular, whereas the pattern of < 4 Hz firing was irregular (Fig. 9*A*). An oscillatory or bursting pattern of firing was never detected. Both the firing pattern (i.e. regular *vs.* irregular) and rate could

be altered by intracellular current injection. Figure 9*B* illustrates that a given neuron could have both regular and irregular firing patterns, and the expression of these patterns depended on the firing rate.

DISCUSSION

Homogeneity vs. heterogeneity of membrane properties

One of the major goals of the present study was to test the hypothesis that the SCN neurons receiving optic nerve input are electrophysiologically homogeneous. These neurons did appear relatively homogeneous in terms of action potential waveform, evoked spike train and its associated properties, membrane time constant and input resistance. With respect to some other intrinsic membrane properties (i.e. inward rectification and LTS potentials), however, the neurons appeared heterogeneous. The heterogeneity could reflect different physiological states manifested as a function of circadian time. This notion, however, is not consistent with the finding that different neurons recorded at the same time of day were heterogeneous, and no apparent relation was detected between a given membrane property and the time of recording. Taken together, our results do not support the hypothesis that the SCN neurons form a homogeneous group electrophysiologically. Neither do they support the idea that the neurons can be classified into a few distinct cell types, such as the cells in the paraventricular nucleus (Hoffman, Tasker & Dudek, 1991; Tasker & Dudek, 1991) or ventromedial hypothalamus (Minami, Oomura & Sugimori, 1986*a*, *b*). Perhaps, the neuronal population consists of several subgroups, and the inherent variability in this type of recording obscured the differences.

Extracellular single-unit studies have reported that the retinorecipient (as well as other) cells in the SCN exhibit different firing patterns (Shibata *et al.* 1984; Cahill & Menaker, 1989*a*), which may be interpreted to mean that the cells have heterogeneous intrinsic membrane properties. The present results suggest that the firing pattern of a given retinorecipient neuron is not an absolute neuronal signature, but more or less a function of firing rate. The different firing patterns of retinorecipient cells could be due to the membrane potential-dependent expression of different subsets of intrinsic membrane properties. More systematic studies considering firing rate might be needed to elucidate the potential link between firing patterns and particular intrinsic membrane properties.

Action potentials

The action potentials recorded in this study looked similar to those of SCN neurons recorded in coronal slices by Wheal & Thomson (1984), except that the action potentials of our neurons had significantly smaller amplitude than theirs (58·2 *vs.* 83·8 mV, $P < 0·001$). This is not likely to be due to poorer impalements, since our neurons had significantly higher input resistance (301 *vs.* 147 MΩ, $P < 0·001$) and longer membrane time constant (11·4 *vs.* 6·7 ms, $P < 0·001$). The smaller spike amplitude could be related to the observation that our neurons were more depolarized; most of our neurons were spontaneously active and had an estimated resting membrane potential that was less negative than −50 mV, whereas their neurons were normally silent and had an average resting potential of −59 mV.

Alternatively, it may be due to the difference in rat strain (Sprague–Dawley *vs.* Wistar) and/or experimental condition such as the time of recording. Lastly, the two studies may have dealt with different neuronal populations (cells with optic nerve input *vs.* cells without the input).

Spike train and post-train after-hyperpolarization

The SCN neurons receiving optic nerve input had similar spike train properties (i.e. spike inactivation, spike broadening and frequency accommodation) to those SCN neurons recorded in coronal slices (Wheal & Thomson, 1984; their synaptic connection with optic nerve was unknown). In addition, almost all the neurons that did not respond to optic nerve stimulation had similar spike train properties (authors' unpublished observation); however, the lack of response could be due to the possible destruction during slice preparation of the afferent from the optic nerve. These results suggest that the spike train properties are not limited only to the neurons that receive optic nerve input; instead, they may be common properties across all SCN neurons.

The post-train after-hyperpolarizations observed in this study were very similar to those described by Wheal & Thomson (1984) for the SCN neurons recorded in coronal slices. Also, they resembled the post-train after-hyperpolarizations observed in other hypothalamic regions. The after-hyperpolarizations in these sites appear to be caused by Ca^{2+}-dependent (Andrew & Dudek, 1984; Bourque, Randle & Renaud, 1985; Minami *et al.* 1986*b*; Bourque & Brown, 1987) and voltage-dependent K^+ conductances (Minami *et al.* 1986*b*). The Ca^{2+}-dependent K^+ conductance in magnocellular supraoptic neurons has been suggested to contribute to frequency adaptation (Andrew & Dudek, 1984; Bourque *et al.* 1985) and the long-term (seconds) spike inhibition following high-frequency firing that occurs in a variety of physiological contexts (Andrew & Dudek, 1984). The post-train after-hyperpolarizations in retinorecipient cells may have similar ionic mechanisms and functions. The estimated reversal potential of the post-train after-hyperpolarization in this study suggests that K^+ is the underlying current carrier.

Inward rectification

A significant proportion of the rat SCN neurons recorded in this study had time-dependent inward rectification, and some had time-independent rectification. Although not as strong as that seen in other central nervous system neurons, such as cerebellar Purkinje cells (Llinás & Sugimori, 1980*a*, *b*), the inward rectification in SCN neurons was clearly detectable. Sugimori *et al.* (1986) reported that guinea-pig SCN neurons had time-dependent inward rectification, and the degree of rectification was comparable to that in the present study. On the other hand, other investigators reported that rat SCN neurons recorded in coronal slices were usually linear in their *I–V* relations (Wheal & Thomson, 1984) and had little or no inward rectification in the hyperpolarizing direction (Thomson & West, 1990). The apparent inconsistency of these reports with our results may be explained by different methods of data analysis (e.g. degree of quantification of results). Alternatively, the discrepancy might be due to the difference in rat strain, experimental condition or neuronal population.

Inward rectification has been observed in a variety of cells, and different functions have been ascribed to it. In mouse dorsal root ganglion neurons, inward rectification can contribute to the anodal break excitation process (Mayer & Westbrook, 1983), while in sino-atrial node it is a part of the pacemaking apparatus (Brown & DiFrancesco, 1980; Brown, 1982). The present results, demonstrating the relationship of the degree of inward rectification with neuronal excitability and with spontaneous firing rate, suggest that inward rectification might be somehow related to or involved in the control of neuronal firing.

LTS potentials

The LTS potentials recorded in SCN neurons did not appear as pronounced as the LTS potentials recorded in other central nervous system sites such as the thalamus (Jahnsen & Llinás, 1984), inferior olive (Llinás & Yarom, 1981) and areas near the paraventricular nucleus (Poulain & Carette, 1987; Hoffman *et al.* 1991; Tasker & Dudek, 1991). Nevertheless, the LTS potentials exhibited very similar voltage dependencies. In inferior olivary (Llinás & Yarom, 1981) and thalamic neurons (Jahnsen & Llinás, 1984), LTS potentials have been suggested to contribute to the oscillation of the membrane potential (i.e. oscillatory firing behaviour). However, a similar role for the LTS potentials appears less likely in retinorecipient cells, considering that we have not detected an oscillatory firing pattern in these neurons. The result that SCN neurons with and without the capacity to generate LTS potentials were not significantly different in spontaneous firing rate and excitability suggests that, at least in the present experimental condition, the capacity to generate LTS potentials does not significantly affect neuronal firing.

Concluding remarks

The results of the present study suggest that the SCN neurons receiving optic nerve input are relatively homogeneous in several intrinsic membrane properties. The study also provides evidence that these neurons are heterogeneous in some other properties, which may or may not be related to neuronal firing. Whether or not this electrophysiological heterogeneity is correlated with morphological and/or histochemical heterogeneity (van den Pol, 1980; Card & Moore, 1984; van den Pol & Tsujimoto, 1985) remains to be elucidated. Answers to these questions may provide an insight to whether or not individual neurons receiving retinal input have different roles in the photic entrainment of circadian rhythms.

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INTRACELLULAR ELECTROPHYSIOLOGICAL STUDY OF SUPRACHIASMATIC NUCLEUS NEURONS IN RODENTS: INHIBITORY SYNAPTIC MECHANISMS

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SUMMARY

1. The mechanisms responsible for evoked and spontaneous fast inhibitory postsynaptic potentials (IPSPs) in the hypothalamic suprachiasmatic nucleus (SCN) were studied with intracellular recording. SCN neurons, primarily ones identified as receiving excitatory optic nerve input, were recorded in rat and guinea-pig brain slice preparations maintained *in vitro*.

2. In normal medium, electrical stimulation of a site dorsocaudal to the SCN evoked IPSPs from thirty-three of thirty-six neurons. The evoked IPSPs rose to the peak quickly ($8{\cdot}7 \pm 0{\cdot}9$ ms, mean ± S.E.M.: $n = 15$ neurons) and decayed gradually with a time constant of 25 ± 3 ms. Spontaneous IPSPs were present in each of the thirty-six neurons. These IPSPs had a rise-to-peak time of $7{\cdot}2 \pm 1{\cdot}0$ ms ($n = 6$ neurons) and a decay time constant of 14 ± 5 ms.

3. When recorded with potassium acetate-filled electrodes, the evoked and spontaneous IPSPs were hyperpolarizing at resting membrane potential (less negative than -70 mV) and had a reversal potential of around -75 mV. On the other hand, when recorded with potassium chloride-filled electrodes, the IPSPs were depolarizing at membrane potentials more negative than -50 mV and had an estimated reversal potential less negative than spike threshold.

4. Bath application of bicuculline (10–50 μM), a γ-aminobutyric acid$_A$ ($GABA_A$) receptor antagonist, resulted in a complete blockade of both the evoked ($n = 16$) and spontaneous ($n = 13$) IPSPs. The bicuculline effects were reversible, and were not associated with any significant and consistent change in baseline membrane potential or input resistance. The neurons impaled in bicuculline-containing medium ($n = 11$) exhibited neither spontaneous IPSPs nor evoked IPSPs.

5. In some neurons bicuculline-resistant hyperpolarizing potentials, which were similar to the fast IPSPs in time course, occurred spontaneously or were evoked by electrical stimulation of the optic nerve or the dorsocaudal site. A fast prepotential always preceded the hyperpolarizing potential, and hyperpolarizing currents blocked these events, indicating that they were not synaptic in origin. No slow IPSPs were detected.

6. The results suggest that fast IPSPs from non-retinal afferents exist in virtually

all SCN neurons receiving excitatory retinal input, and that $GABA_A$ receptors associated with Cl^- channels mediate the fast IPSPs.

INTRODUCTION

In mammals the major pacemaker for circadian rhythms appears to be located in the hypothalamic suprachiasmatic nuclei (SCN) (for reviews, see Takahashi & Zatz, 1982; Moore, 1983; Turek, 1985; Meijer & Rietveld, 1989). Several studies have pointed to the possibility that γ-aminobutyric acid (GABA), via its action at the level of the SCN, may participate in mammalian circadian time keeping. The SCN contains GABAergic neurons (Tappaz, Brownstein & Kopin, 1977; Card & Moore, 1984; van den Pol & Tsujimoto, 1985; van den Pol, 1986; Okamura, Bérod, Julien, Geffard, Kitahama, Mallet & Bobillier, 1989), and nearly 50% of synaptic boutons in the SCN are immunoreactive for GABA (Decavel & van den Pol, 1990). Furthermore, exogenously applied GABA and its related agents alter the single-unit activity of a significant proportion of SCN neurons (Shibata, Liou & Ueki, 1983; Liou & Albers, 1990; Liou, Shibata, Albers & Ueki, 1990; Mason, Biello & Harrington, 1991).

Agents that alter GABAergic neurotransmission have been reported to affect the circadian rhythms of rodents. Single intraperitoneal injections of triazolam, a short-acting benzodiazepine, induce a permanent phase shift in free-running behavioural and endocrine rhythms (for review, see Turek & Van Reeth, 1988), while bicuculline, a $GABA_A$ receptor antagonist, blocks the light-induced phase delays of free-running locomotor rhythm (Ralph & Menaker, 1985). It is not clear where and how these GABAergic drugs act to influence the circadian time-keeping system. A recent study by Johnson, Smale, Moore & Morin (1988) suggests that the lateral geniculate nucleus of the thalamus might be the critical site for the triazolam-induced phase shifts. On the other hand, more recent studies by Smith, Inouye & Turek (1989) and by Smith, Turek & Slater (1990) suggest that the SCN might be an important site for GABAergic drug action. According to their reports, microinjection of muscimol, a selective $GABA_A$ receptor agonist, into the SCN led to a permanent phase advance or delay of free-running locomotor rhythms of hamster; the phase advance was selectively blocked by the $GABA_A$ receptor antagonists, bicuculline and picrotoxin, but not by the $GABA_B$ receptor antagonist, phaclofen.

Several studies have suggested a neurotransmitter role for GABA in the hypothalamus. The evidence includes the presence of numerous GABAergic boutons and axons in various hypothalamic nuclei (Tappaz *et al.* 1977; Decavel & van den Pol, 1990), and the sensitivity to $GABA_A$ receptor antagonist of inhibitory postsynaptic potentials (IPSPs) recorded in some hypothalamic nuclei (Randle, Bourque & Renaud, 1986; Tasker & Dudek, 1988; Hoffman, Kim, Gorski & Dudek, 1990). With regard to the SCN, strong morphological evidence exists for a neurotransmitter role for GABA, but solid physiological evidence is lacking.

The present study was designed to provide physiological evidence that GABA, acting on $GABA_A$ receptors to increase chloride conductance, is the dominant fast inhibitory transmitter in the SCN. In this study, we tested whether bicuculline, a $GABA_A$ receptor antagonist, blocked the spontaneous and evoked IPSPs recorded in the SCN. Since the results of our previous study indicated that optic nerve input to

SCN neurons is excitatory (Kim & Dudek, 1991), we attempted to evoke IPSPs by stimulating other, non-retinal afferents. Here we present intracellular electrophysiological evidence that $GABA_A$ receptors mediate fast IPSPs in the SCN and that fast IPSPs are present in the vast majority of, if not all, SCN neurons. A preliminary account of these results has been published (Kim & Dudek, 1990).

METHODS

Animals

Male Sprague–Dawley rats ($n = 26$, 120–350 g) and guinea-pigs ($n = 5$, 150–450 g) purchased from Charles River Company (USA) were housed in a temperature controlled vivarium (22–23 °C) under a 12 h light/12 h dark cycle (light on at 07.00 h, Pacific Time) for at least 1 week (mostly more than 2 weeks) prior to use.

Preparation and maintenance of hypothalamic slices

In the morning (after 07.00 h) of the day of the experiment each animal was decapitated with a guillotine under sodium pentobarbitone (I.P., 100 mg/kg wt) anaesthesia. Immediately after decapitation, the brain was removed from the skull and placed in ice-cold physiological saline (composition in mM: 124 NaCl, 1·4 NaH_2PO_4, 3 KCl, 2·4 $CaCl_2$, 26 $NaHCO_3$, 1·3 $MgSO_4$, 11 glucose). After approximately 1 min, the brain was trimmed to a small block to contain the hypothalamus and optic nerves. Then, using a vibroslicer (Campden Instruments, UK), two 500 μm thick parasagittal slices containing the SCN and optic nerve were cut from the block. In one case (rat), a coronal slice was cut from the block. The slices were immediately transferred to and maintained in an interface-type recording chamber which was constantly perfused with warmed (32–35 °C) physiological saline at 0·7–1·0 ml/min. In some experiments, bicuculline methiodide (Sigma, USA) was added to the perfusing medium. A humidified atmosphere of 95% O_2 and 5% CO_2 was maintained above the slice throughout the experiments. Electrophysiological experiments were begun at least 2 h after the slice preparation.

Intracellular recordings and electrical stimulation

Intracellular micropipettes were filled with either 2 M potassium acetate or 2·3 M potassium chloride (90–360 MΩ). Current-clamp recordings were obtained using a high-impedance amplifier with a bridge circuit (Axoclamp-2A, Axon Instruments, USA). Bipolar electrodes were used to stimulate the optic nerve ($\leqslant$ 0·8 mA, 0·5 ms) and a site dorsocaudal to the SCN ($\leqslant$ 1·0 mA, 0·5 ms). The electrode placement in the dorsocaudal aspect varied among different experiments (i.e. more dorsal in some, more caudal in others). The distance of the electrode from the SCN was also variable among experiments (range, 0·5–3 mm).

RESULTS

The data presented in this paper were from forty-eight SCN neurons (thirty-six rat and twelve guinea-pig neurons), which were recorded between 10.00 h and 21.00 h and whose mean ($\pm$S.E.M.) action potential amplitude (measured from spike threshold) and input resistance (estimated from the slope of the linear portion of current–voltage plot) were 60 $\pm$ 1 mV ($n = 28$) and 255 $\pm$ 27 MΩ ($n = 28$), respectively. The spontaneous firing rate (0–19 Hz) did not appear to correlate with the time of recording. In terms of the properties of postsynaptic potentials, rat and guinea-pig neurons were not obviously different, therefore, the data from the two species were combined.

Characteristics of evoked and spontaneous IPSPs

In bicuculline-free medium electrical stimulation of optic nerve evoked an excitatory postsynaptic potential (EPSP) in a subpopulation of SCN neurons (~ 30–40% of > 100 neurons). However, stimulation of a site dorsocaudal to the SCN

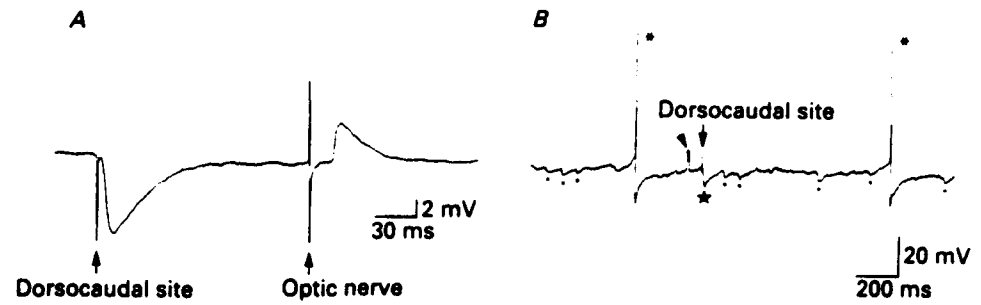

Fig. 1. Convergence of the excitatory optic nerve and inhibitory non-optic nerve inputs to SCN neurons. *A*, an IPSP and an EPSP evoked by stimulation (arrow) of a site dorsocaudal to the SCN and optic nerve, respectively. The trace is an average of twenty responses. *B*, individual trace showing spontaneous (●) and evoked (★) IPSPs recorded from another neuron that responded to optic nerve stimulation with an EPSP (not shown). A 10 mV, 10 ms calibration pulse (arrow-head) preceded dorsocaudal stimulation (arrow). Action potentials are labelled with asterisks. Cells were recorded with potassium acetate-filled electrodes and current-clamped to −61 mV (*A*) and −52 mV (*B*). Unless stated otherwise, all the records presented in the following figures were obtained with potassium acetate-filled electrodes.

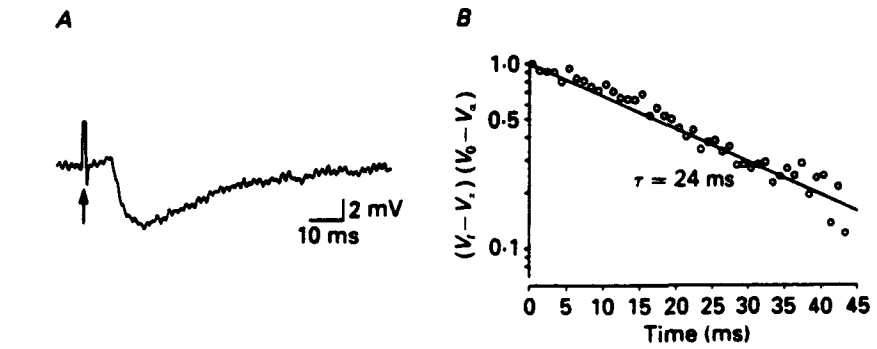

Fig. 2. The IPSPs decayed exponentially. *A*, an IPSP evoked by dorsocaudal stimulation (arrow). *B*, for the decay of the evoked IPSP in *A*, the values of $(V_t-V_r)/(V_0-V_r)$, taken at 1 ms intervals and integrated over ±0·2 ms, were plotted against time, where V_t is the voltage at time t, V_0 is the voltage at the beginning of the decay and V_r is the voltage at the baseline. A linear regression line was fitted to the plot to calculate the time constant (τ = −0·4343/slope of the line; Rall, 1969). The cell was at resting membrane potential (−58 mV).

evoked an IPSP in most of the neurons examined (thirty-three of thirty-six neurons). Figure 1*A* illustrates that neurons that responded to optic nerve stimulation with an EPSP (n = 26) also responded to dorsocaudal site stimulation with an IPSP (n = 23).

Across different neurons the latency of the evoked IPSPs (measured at 50% of the IPSP peak) varied significantly (range, 5·5–13·5 ms), presumably due to differences in the distance between the recording and stimulation sites. Within a given neuron, however, the latency was virtually constant; the mean latency had a standard deviation of 0·47 ms on average (range of standard deviation across seven neurons, 0·17–0·69 ms; 7–16 IPSPs per neuron). In every neuron tested (n = 5), the IPSPs could follow 10 or 20 Hz stimulation.

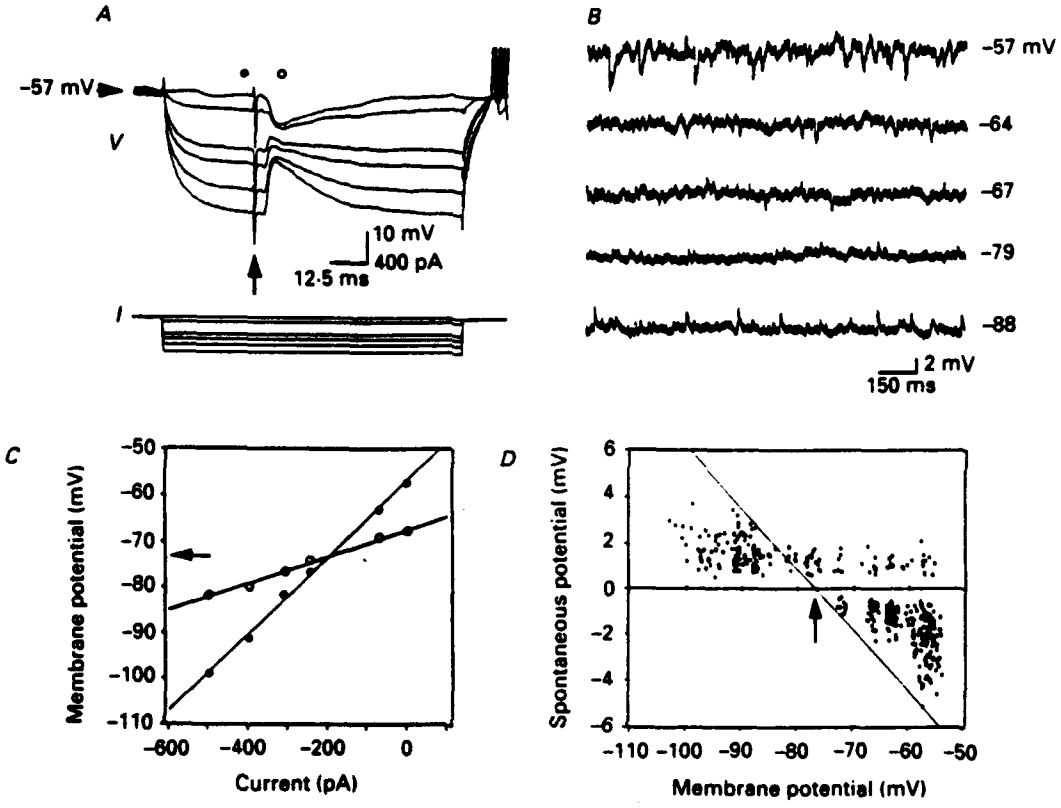

Fig. 3. IPSP reversal potential. *A*, IPSPs evoked by dorsocaudal stimulation (arrow) at the peak of the electrotonic potentials (*V*) elicited by injecting hyperpolarizing current pulses of various amplitudes (*I*). Each voltage trace is an average of three to six responses. *B*, spontaneous IPSPs recorded at the membrane potentials indicated to the right of each trace. These IPSPs were from the same neuron as *A*. Both the evoked and spontaneous IPSPs were hyperpolarizing at membrane potentials less negative than −70 mV, and depolarizing at membrane potentials more negative than −80 mV. *C*, current–voltage plots constructed from the data in *A*. Voltage measurements were obtained immediately before the stimulation (●) and at the peak of the IPSP (○). The slopes of the linear regression lines fitted to the plots were compared to estimate the change in input resistance during the IPSP; input resistance decreased by about 67% at the peak of the IPSP. The membrane potential where the regression lines cross (arrow, −73 mV) was taken as the reversal potential for the IPSP. *D*, the amplitude of spontaneous IPSPs in *B* was plotted against membrane potential. Other spontaneous IPSPs not shown in *B* were also included in the plot. The points in the upper right quadrant of this plot correspond presumably to spontaneous EPSPs. The continuous line shows the upper limit of the variation in the IPSP amplitude. The intercept of this line with the abscissa (arrow, −77 mV) was taken as the reversal potential for the spontaneous IPSPs.

The evoked IPSPs rose to the peak in 3·9–18·6 ms (8·7 ± 0·9 ms, $n = 15$ neurons), and decayed gradually over 50–150 ms (Fig. 2*A*). In most cases, the decay could be fitted reasonably well with a single time constant (25 ± 3 ms; range 12–52 ms, $n = 15$ neurons, Fig. 2*B*). Neither the rise-to-peak time nor the decay time constant of the IPSPs appeared to vary with the time of day or season. At the peak of the IPSP, input resistance decreased by 48 ± 6% (range 31–67%; $n = 6$, Fig. 3*C*).

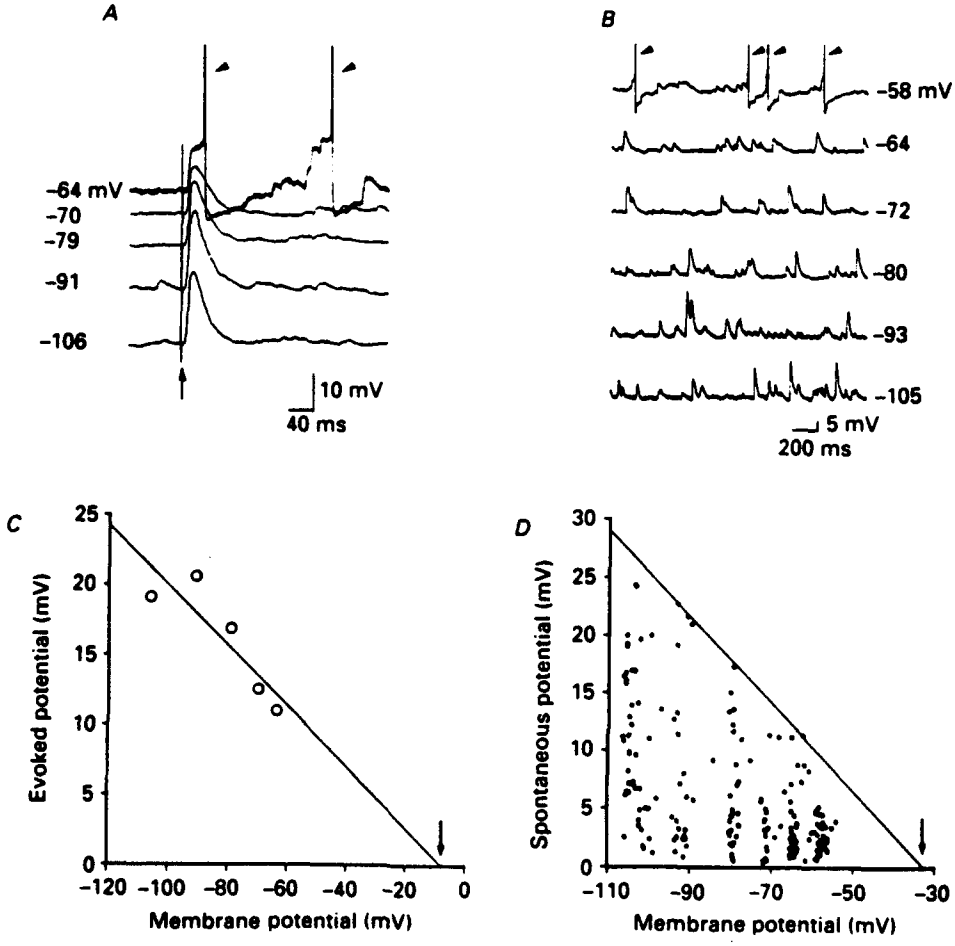

Fig. 4. Reversal of the polarity of IPSP by intracellular Cl^- loading. *A*, depolarizing postsynaptic potentials evoked by stimulation of the dorsocaudal site (arrow) at the membrane potentials indicated to the left of each trace. This cell was recorded with potassium chloride-filled electrodes. All of the traces except the top one are an average of eight to fifteen responses. The action potentials (arrow-heads) in the top trace are truncated. *B*, spontaneous depolarizing postsynaptic potentials recorded at the membrane potentials indicated to the right of each trace. Thee potentials were from the same neuron as in *A*. The postsynaptic potentials recorded in this neuron were always depolarizing at membrane potentials more negative than −50 mV, and could support action potentials (arrow-heads; truncated). *C* and *D*, the amplitudes of the postsynaptic potentials in *A* and *B* were plotted against membrane potential in *C* and *D*, respectively. For the plot in *D*, extra data points other than those shown in *B* were also included. A linear regression line was fitted to the plot in *C* to estimate the reversal potential (arrow: −7 mV) of the evoked postsynaptic potential. The continuous line in *D* shows the upper limit of the variation in the spontaneous postsynaptic potential amplitude. The intercept of this line with abscissa (arrow: −32 mV) was taken as the reversal potential.

Spontaneous IPSPs occurred in all thirty-seven neurons recorded in bicuculline-free medium (Fig. 1*B*), and their frequency of occurrence was normally high (> 10 IPSPs/s in eighteen out of twenty-five neurons). Similar to the evoked IPSPs, the spontaneous IPSPs rose to their peaks quickly and decayed gradually. For six

neurons where ten to thirty spontaneous IPSPs (≥ 2 mV) were analysed, the rise-to-peak time was $7{\cdot}2 \pm 1{\cdot}0$ ms (range 3·9–11·2 ms) and the decay time constant was 14 ± 5 ms (range 8–20 ms).

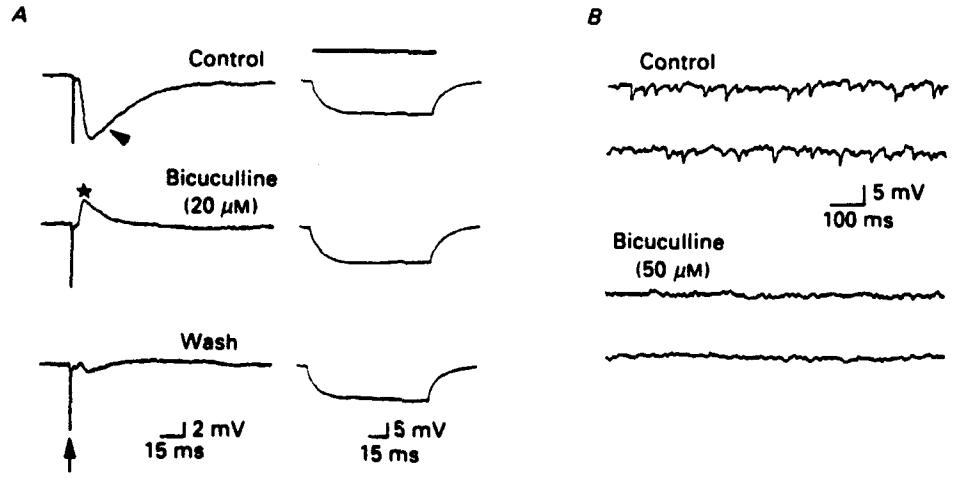

Fig. 5. Blockade of IPSPs by bicuculline. *A*, synaptic responses (left panel) evoked by stimulation (arrow) of the dorsocaudal site and electrotonic potentials (right panel) elicited by injecting a hyperpolarizing current pulse (−50 pA, 100 ms; continuous line) under each experimental condition. Each trace is an *average of ten to twenty responses*. The blockade by bicuculline (20 μM) of the evoked IPSP (arrow-head) revealed an EPSP (★), which was shunted by the IPSP during control condition. Wash-out of bicuculline resulted in disappearance of the EPSP and reappearance of the IPSP of a smaller magnitude. Note the lack of a slow hyperpolarizing component (i.e. slow IPSP) in any of the experimental conditions. Also, note that bicuculline had no significant effect on the baseline input resistance. *B*, individual traces showing spontaneous IPSPs (upper panel) and the effects of bicuculline (50 μM) on these IPSPs (lower panel). The data in *A* and *B* were obtained from two different neurons, current-clamped to −61 (*A*) and −57 mV (*B*).

Dependence of the IPSP reversal potential on Cl^- concentration gradient

When recorded with potassium acetate-filled electrodes, the evoked and spontaneous IPSPs were hyperpolarizing at resting membrane potential (less negative than −70 mV) and depolarizing at membrane potentials more negative than −80 mV (Fig. 3*A* and *B*). The estimated reversal potentials for the evoked and spontaneous IPSPs (Fig. 3*C* and *D*) were -75 ± 2 mV (range −70 to −83 mV, $n = 6$) and -74 ± 1 mV (range −70 to −77 mV, $n = 4$), respectively. These values are close to the Cl^- equilibrium potential (−75 mV) calculated with the assumption that intracellular Cl^- concentration is 8 mM (McCormick, 1990), suggesting that Cl^- may be the current carrier for the IPSPs.

If the IPSPs are due to an increase in Cl^- conductance, then changes in the Cl^- concentration gradient across the cell membrane should be accompanied by changes in the IPSP reversal potential. By recording with potassium chloride-filled electrodes ($n = 7$), cells were loaded with Cl^-, which altered the Cl^- concentration gradient. Immediately after impalement of the cells with these electrodes, hyperpolarizing postsynaptic potentials similar to the IPSPs recorded with potassium acetate-filled

electrodes were present (data not shown). Over 1–2 min, however, depolarizing postsynaptic potentials (Fig. 4*A* and *B*) gradually replaced the hyperpolarizing postsynaptic potentials, presumably due to gradual increase in the intracellular Cl^- concentration. The estimated reversal potential for the depolarizing postsynaptic

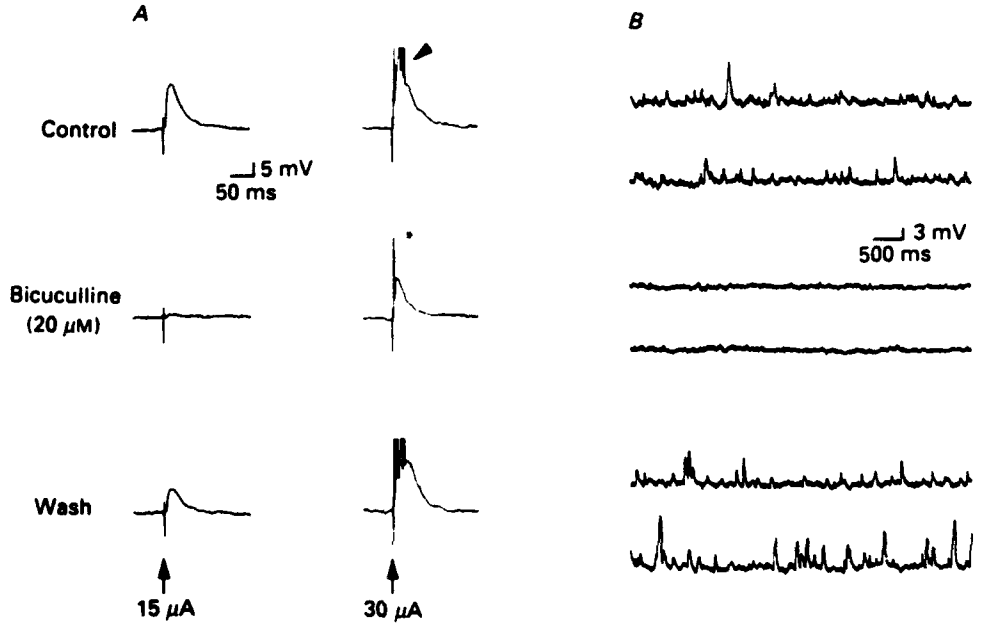

Fig. 6. Blockade of the depolarizing IPSPs (potassium chloride-filled electrode) by bicuculline. *A*, synaptic responses evoked under each experimental condition by stimulation (arrow) of the dorsocaudal site at 15 (left panel) and 30 μA (right panel). Each trace is an average of six responses. Note that, under the control condition, the 30 μA stimulation triggered a burst of action potentials (arrow-head; truncated). Also, note that the response from 15 μA stimulation had very little bicuculline-resistant component while the one from 30 μA stimulation had a significant bicuculline-resistant component, which generated an action potential (asterisk; truncated). *B*, individual traces obtained under each experimental condition from the same neuron in *A*, showing the effects of bicuculline on spontaneous depolarizing postsynaptic potentials. The bicuculline effects both in *A* and *B* were not associated with any significant changes in the baseline input resistance (data not shown). The cell was current-clamped to −77 mV.

potentials was always less negative than spike threshold ($n = 4$ neurons, Fig. 4*C* and *D*). These results further support the hypothesis that Cl^- is the current carrier for the IPSPs in the SCN.

Blockade of IPSPs by bicuculline

Bath-applied bicuculline (10–50 μM) blocked both the evoked ($n = 13$) and spontaneous ($n = 10$) IPSPs recorded with potassium acetate-filled electrodes (Fig. 5). The blockade of the evoked IPSP was accompanied by either an emergence of an EPSP (Fig. 5*A*) or a significant increase in the amplitude of the EPSP that sometimes preceded the IPSP. The blockade of IPSPs was complete within

10–15 min after the onset of bicuculline application, and was reversed by wash of bicuculline; in neurons where a 15–90 min wash was possible without losing the impalement, a partial to full recovery from the bicuculline effects was clearly achieved for both evoked (6 out of 6 neurons) and spontaneous (4 out of 6 neurons) IPSPs. The bicuculline effects were not associated with any significant changes in the

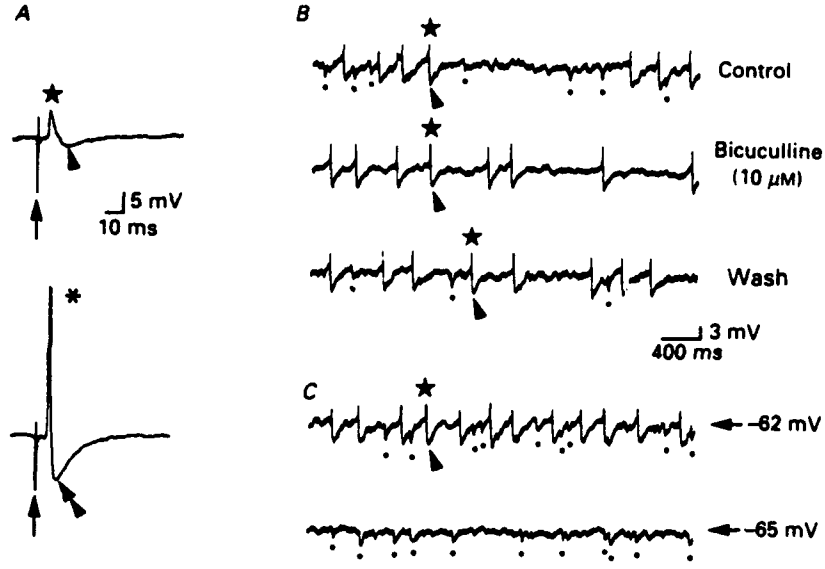

Fig. 7. Fast prepotential and its associated hyperpolarizing after-potential resembling fast IPSP. *A*, fast prepotential (★) and hyperpolarizing after-potential (arrow-head) evoked by dorsocaudal stimulation (arrow) in 50 μM bicuculline (upper panel). Occasionally, the fast prepotential could trigger an action potential (asterisk: truncated) (lower panel). The hyperpolarizing after-potential associated with the action potential (double arrow-heads) was of a greater magnitude. The cell was current-clamped to −74 mV. *B*, effect of bicuculline on spontaneous fast prepotentials and their associated hyperpolarizing after-potentials. Unlike spontaneously occurring fast IPSPs (●), the fast prepotentials (★) and the hyperpolarizing after-potentials (arrow-head) were not blocked by bicuculline. The cell was current-clamped to −63 mV. *C*, effect of membrane potential change on the fast prepotentials and the hyperpolarizing after-potentials. In bicuculline-free condition, the cell was current-clamped to the membrane potentials indicated to the right of each trace. Unlike fast IPSPs, fast prepotentials and the hyperpolarizing after-potentials were absent at membrane potentials more negative than −65 mV. The traces in *B* and *C* were from the same neuron.

baseline input resistance of postsynaptic neurons (105 ± 10% of the control, $n = 6$, Fig. 5*A*) and, in most cases, the baseline membrane potential was steady during bicuculline application; in a few cases, however, a depolarization of 5–10 mV followed bicuculline application. The depolarizing postsynaptic potentials recorded with potassium chloride-filled electrodes were also sensitive to bicuculline (20–50 μM). The evoked depolarizing postsynaptic potentials were partially blocked by bicuculline and had a bicuculline-resistant component, which was presumably an EPSP (Fig. 6*A*, $n = 3$). The spontaneous depolarizing postsynaptic potentials were

completely blocked by bicuculline (Fig. 6*B*, $n = 3$). Again, the effects of bicuculline were reversible and not associated with any significant change in baseline membrane potential or input resistance.

The neurons impaled with potassium acetate-filled electrodes in the continued presence of bicuculline (50 μM) in the perfusing medium never exhibited either evoked or spontaneous IPSPs ($n = 11$). In bicuculline, stimulation of the dorsocaudal site (as well as optic nerve) evoked only an EPSP, an action potential and/or a fast prepotential. The evoked fast prepotentials were occasionally followed by hyperpolarizing after-potentials with a time course that resembled the fast IPSPs (Fig. 7*A*). Hyperpolarizing after-potentials also followed the spontaneously occurring fast prepotentials observed in three neurons (Fig. 7*B* and *C*). Although bicuculline did not affect these potentials (Fig. 7*B*), hyperpolarization of the neuron beyond a certain point completely arrested the fast prepotentials and the associated hyperpolarizing after-potentials (Fig. 7*C*), indicating a non-synaptic nature of their origin. In the present study, we did not detect any slow IPSPs.

DISCUSSION

$GABA_A$ receptors mediate the IPSPs in the SCN

Numerous anatomical studies have suggested a neurotransmitter role for GABA in the SCN. The results of the present study provide physiological evidence for such a role. In addition, our data suggest that $GABA_A$ receptors mediate inhibitory transmission in the SCN. This conclusion is consistent with the one drawn by Thomson & West (1990) from their preliminary results that ionophoretically applied bicuculline blocked spontaneous IPSPs in the SCN. Also, the conclusion is supported by recent reports by Liou *et al.* (1990) and Mason *et al.* (1991) that bicuculline antagonized the depression of neuronal firing in the SCN caused by exogenously applied GABA, $GABA_A$ receptor agonist muscimol and/or benzodiazepines. The current findings that bicuculline-sensitive spontaneous IPSPs were present in all the neurons recorded in normal medium and that stimulation of a site dorsocaudal to the SCN also evoked bicuculline-sensitive IPSPs from most of these neurons are in line with the observation made by Decavel & van den Pol (1990) in their immunocytochemical study that all the SCN neurons examined were innervated by GABA-positive axons. Taken together, these results strongly support the hypothesis that GABA, acting on $GABA_A$ receptors, is the dominant inhibitory neurotransmitter in the SCN.

Type of IPSPs in the SCN

Both evoked and spontaneous IPSPs had a fast rising phase, and their decay could be fitted with a single time constant in most cases. This observation, coupled with the findings that the IPSPs were associated with a postsynaptic increase in Cl^- conductance and completely blocked by the $GABA_A$ receptor antagonist bicuculline, strongly suggests that the IPSPs were of the fast type, and not mixed with slow IPSPs (see below). The IPSPs observed in our study were similar to those recorded from supraoptic nucleus neurons in hypothalamic explants (Randle *et al.* 1986), in terms of the time course, dependence on Cl^- conductance and sensitivity to

bicuculline. $GABA_A$ receptors may thus mediate fast inhibitory synaptic transmission throughout the hypothalamus.

Lack of slow IPSPs in the SCN

$GABA_B$ receptor-mediated, slow IPSPs have been documented in many brain regions (Dutar & Nicoll, 1988; Hasuo & Gallagher, 1988; Soltesz, Haby, Leresche & Crunelli, 1988; Karlsson & Olpe, 1989; McCormick, 1989). However, the results of our study do not provide support for the presence of slow IPSPs in the SCN, because, as mentioned above, the blockade of IPSPs by bicuculline was complete without leaving any obvious bicuculline-resistant, slow hyperpolarizing component. The bicuculline-resistant hyperpolarizing after-potentials that followed fast prepotentials were fast in time course, and appeared to be non-synaptic events (i.e. not slow IPSPs). The lack of any significant and consistent effects of bicuculline at the concentrations used here (10–50 μM) on the passive membrane properties of postsynaptic neurons suggests that the blockade of IPSPs by bicuculline was specific. Comparable concentrations of bicuculline were reported to block the fast IPSPs recorded from the supraoptic (Randle *et al.* 1986) and paraventricular (Tasker & Dudek, 1988) nuclei in the hypothalamus. In the dorsal lateral geniculate nucleus of the thalamus, 50 μM bicuculline was shown to block fast, but not slow, IPSPs (Soltesz, Lightowler, Leresche & Crunelli, 1989). This suggests that bicuculline at concentrations $\leqslant 50\ \mu$M is selective for $GABA_A$ receptors.

Several explanations need to be considered for the lack of slow IPSPs in these recordings. The inability to detect slow IPSPs might have been due to the low signal-to-noise ratio associated with the high resistance sharp electrodes used. However, when slow IPSPs were present in other neurons, they could be recorded with similar high resistance electrodes. The employed stimulus intensity (up to 1 mA) may not have been sufficient to evoke slow IPSPs (Dutar & Nicoll, 1988). It is also possible that the perfusion rate was so high that effective concentrations of GABA for $GABA_B$ receptor activation (i.e. for slow IPSP) could not be achieved with the electrical stimulation. These explanations seem unlikely, because lower intensity stimulation ($< 0{\cdot}1$ mA) at a similar perfusion rate could evoke slow IPSPs in slices from other parts of the brain (Y. I. Kim & F. E. Dudek, unpublished observation). The lack of slow IPSPs might be due to the particular site of electrical stimulation (i.e. due to limited stimulation of the afferents that would evoke only fast IPSPs) or to the fact that the electrical stimulation of the presynaptic sites was not equivalent to physiological synaptic activation.

The absence of slow IPSPs in the SCN could be viewed as evidence against the existence of postsynaptic $GABA_B$ receptors. However, it would not exclude the possibility that functional $GABA_B$ receptors exist at other locations, such as extrasynaptic sites or serotonergic axonal elements (François-Bellan, Héry, Faudon & Héry, 1988; Bosler, 1989). It might be through these receptors that the $GABA_B$ receptor agonist, baclofen, depressed the neuronal discharge in the rat SCN (Liou *et al.* 1990) and phase-advanced the hamster circadian rhythm of locomotor activity (Smith *et al.* 1990).

Does GABAergic input arise locally?

The present data indicate that afferents other than the optic nerve are the source of inhibitory GABAergic input to SCN neurons. The techniques employed in this study, however, do not allow determination of whether the input arises locally from GABAergic cells in the SCN (Tappaz *et al.* 1977; Card & Moore, 1984; van den Pol & Tsujimoto, 1985; van den Pol, 1986; Okamura *et al.* 1989) or from cells outside the SCN. That the evoked IPSPs had a virtually constant onset latency and followed stimuli presented at 10 or 20 Hz only suggests that the GABAergic input is monosynaptic. Further studies with other techniques, such as glutamate microdrop application (Christian & Dudek, 1988), may provide more direct answers to these questions.

Role of GABAergic input to SCN neurons

Several lines of research have suggested that GABA is an important neurotransmitter in circadian time keeping. A series of studies by Ralph & Menaker (1985, 1986 and 1989) has provided evidence that light-induced phase shifts of the circadian locomotor rhythm of hamsters involve GABAergic regulation. Conceivably, the SCN neurons receiving optic nerve input (i.e. light) might be an important site for the GABAergic regulation. The direct GABAergic innervation of the retinorecipient neurons demonstrated in the present study supports this idea. Nevertheless, the exact functional significance of the GABAergic transmission in SCN neurons remains to be determined. As argued by Smith *et al.* (1990), the GABAergic transmission in the SCN may not be normally involved in circadian timing system.

The finding that spontaneous IPSPs occurred frequently in most of the neurons studied differs from the data of Wheal & Thomson (1984) that spontaneous synaptic activity was absent in SCN neurons, but agrees with the more recent report by Thomson & West (1990) that spontaneous IPSPs occurred frequently. The spontaneous IPSPs occurring at high rates suggest that significant ongoing synaptic inhibition is present in the SCN maintained *in vitro*. This pronounced synaptic inhibition may also exist in *in vivo* conditions and contribute significantly in controlling the baseline membrane potential of postsynaptic neurons. The depolarization (5–10 mV) that followed the bicuculline blockade of spontaneous IPSPs in some neurons supports this hypothesis.

In summary, fast IPSPs in SCN neurons are associated with a postsynaptic increase in Cl^- conductance, and are blocked by bicuculline. All the SCN neurons examined, including those identified as receiving excitatory optic nerve input, had fast IPSPs; this suggests that $GABA_A$ receptor-mediated, fast inhibitory synaptic input is present in most, if not all, SCN neurons.

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Neurobiology

Neuronal synchronization without calcium-dependent synaptic transmission in the hypothalamus

(suprachiasmatic nucleus/intercellular communication/nonsynaptic mechanism/circadian rhythm/patch clamp)

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ABSTRACT **A critical question in understanding the mammalian brain is how populations of neurons become synchronized. This is particularly important for the neurons and neuroendocrine cells of the hypothalamus, which are activated synchronously to control endocrine glands and the autonomic nervous system. It is widely accepted that communication between neurons of the adult mammalian brain is mediated primarily by Ca^{2+}-dependent synaptic transmission. Here we report that synchronous neuronal activity can occur in the hypothalamic suprachiasmatic nucleus without active Ca^{2+}-dependent synaptic transmission. Simultaneous extracellular recordings of neuronal activity in the suprachiasmatic nucleus, which contains the mammalian biological clock, confirmed a circadian rhythm of *synchronized* activity in hypothalamic slices. Ca^{2+}-free medium, which blocks chemical synaptic transmission and increases membrane excitability, produced periodic and *synchronized* bursts of action potentials in a large population of suprachiasmatic nucleus neurons with diverse firing patterns. *N*-Methyl-D-aspartic acid, non-*N*-methyl-D-aspartic acid, and γ-aminobutyric acid type A receptor antagonists had no effect on burst synchrony. Whole-cell patch-clamp recordings confirmed that the Ca^{2+}-free solution blocked evoked postsynaptic potentials and that the mixture of antagonists blocked the remaining spontaneous postsynaptic potentials. Therefore, mechanisms other than Ca^{2+}-dependent synaptic transmission can synchronize neurons in the mammalian hypothalamus and may be important wherever neuronal networks are synchronized.**

There is virtually no physiological information on local neuronal interactions in the hypothalamus, even though synchronization of the neuronal and neuroendocrine elements of the hypothalamus is a critical and fundamental process in the neurobiology of hormone secretion and homeostasis. In freely moving animals, the neuronal activity in the suprachiasmatic nucleus (SCN) of the hypothalamus, which contains the mammalian biological clock (1, 2), exhibits a circadian rhythm where peak activity occurs at the middle of the light phase, even in a surgically isolated hypothalamus (3) or *in vitro* brain slice (4–7). Because these data are derived from neuronal populations, they also imply that the electrical activity generating the pacemaker is synchronized. Determining the mechanism(s) underlying this synchronization is critical to an understanding of circadian rhythm generation and neuronal interactions in the hypothalamus. The Ca^{2+}-independent nonsynaptic mechanism(s) of synchronization we describe here in the SCN provides a crucial clue for understanding the cellular processes underlying circadian rhythm generation, since it is quite likely that the circadian rhythm incorporates the same mechanism.

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Most of the studies on neuronal networks in the mammalian brain have been directed toward chemical synaptic transmission, while other nonconventional forms of communication between neurons have been relatively neglected and probably underestimated. Previous studies have suggested that certain areas of the brain (e.g., hippocampus, inferior olive, and locus coeruleus) exhibit synchronization independent of chemical synaptic transmission, but the activity appears to be tightly synchronized (i.e., neuronal activity occurs in a one-for-one manner throughout the population; see *Discussion*). Here we show that neuronal activity in the hypothalamus can be loosely synchronized (i.e., action potentials generally occur together, but with different firing patterns) after chemical synaptic transmission has been blocked. This significantly extends the brain areas in which a nonchemical synaptic mechanism has been shown to synchronize activity of large neuronal populations in the adult mammalian brain.

MATERIALS AND METHODS

Slice Preparation. Coronal hypothalamic slices (350–500 μm) were prepared 2 h before the dark phase from Sprague–Dawley male rats (100–400 g, except 40–85 g for patch-clamp experiments). Rats were obtained from a single colony for circadian rhythm experiments and maintained on a 12-hr light/12-hr dark schedule for ≥14 days. The slices were studied in an interface chamber (33–35°C, in humidified 95% O_2/5% CO_2).

Solutions. For the circadian rhythm experiments (see Fig. 1), slices were bathed in a physiological solution containing 124 mM NaCl, 3 mM KCl, 1.4 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 11 mM glucose, and 26 mM NaH_2CO_3. Control solutions in the other experiments (see Figs. 2 and 3) were similar to those for Fig. 1 except that the NaCl concentration was 134 mM, 10 mM Hepes was used as a buffer, and 8 mM NaOH was added to adjust to pH 7.4. In the Ca^{2+}-free medium, $MgCl_2$ was substituted for $CaCl_2$, and 0.1 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, a specific Ca^{2+} chelator, was added. Blockade of *N*-methyl-D-aspartic acid (NMDA), non-NMDA, and γ-aminobutyric acid type A ($GABA_A$) receptors was achieved by bath application of 100 μM DL-2-amino-5-phosphonopentanoic acid, 50 μM 6,7-dinitroquinoxaline-2,3-dione, and 50 μM bicuculline.

Extracellular Recordings. Multiple-unit activity (MUA) was recorded simultaneously with two metal electrodes (90% platinum/10% iridium wire coated with teflon, diameter = 76 μm). The signals were amplified with a differential amplifier

Abbreviations: SCN, suprachiasmatic nucleus; NMDA, *N*-methyl-D-aspartic acid; $GABA_A$, γ-aminobutyric acid type A; MUA, multiple-unit activity; PSP, postsynaptic potential.

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(bandwidth filter = 0.3–3 kHz). Discharges above a threshold (signal-to-noise ratio ≥ 1.5–2.0:1) were counted in 5-min bins and then averaged in 1-h bins. Single-unit activity was recorded by using glass micropipettes filled with 1 M NaCl (resistance = 30–40 MΩ).

Whole-Cell Patch-Clamp Recordings. Glass pipettes were filled with solution containing 120 mM potassium gluconate, 1 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 4 mM MgATP, 5 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid and adjusted to pH 7.2 with KOH. Electrode resistance before seal formation was 4–7 MΩ. Extracellular stimulation applied through a platinum/iridium bipolar electrode to a site 2–3 mm dorsal to the SCN (0.2–1.6 mA and 0.5 ms) evoked an excitatory postsynaptic potential (PSP) starting after a 2- to 5-ms delay with 5–16 mV peak amplitude and duration of 60–120 ms. Input resistance was 657 ± 80 MΩ (mean ± SE; n = 12; range of 350–1150 MΩ) at membrane potentials of −70 to −90 mV. During PSP blockade, no reduction was observed in input resistance (n = 5), indicating that PSPs were not shunted by a leak in the membrane.

RESULTS

Simultaneous recordings of MUA with two electrodes positioned on the slice surface were made for 25 h. One electrode was located in the midventral SCN and the other in the dorsal SCN (100- to 250-μm separation). These recordings confirmed that a circadian rhythm can be obtained in an isolated slice (Fig. 1*A*); peak activity occurred at 06:29 ± 0:26 h circadian time (mean ± SE; n = 6; lights on from 00:00 to 12:00 h circadian time), in agreement with other *in vivo* and *in vitro* studies (3–7). The correlation between the two hourly MUA counts across the circadian rhythm in the same SCN was high (r = 0.99 for Fig. 1*B*; r = 0.73, 0.61, 0.87, and 0.93 in other cases; df = 23; $P < 0.0015$), thus confirming synchrony in circadian neuronal activity (Fig. 1*B*).

To test whether chemical synaptic transmission is required for synchronization of SCN neurons, we bathed hypothalamic slices in Ca^{2+}-free solution, which blocks evoked chemical PSPs (8–10) and also increases membrane excitability (11). After 2.5–5.5 h, periodic bursts of neuronal activity appeared in the SCN. Simultaneous MUA recordings from two locations within one SCN revealed that the bursts always occurred simultaneously (n = 11; Fig. 2*A*). Bursts were never synchronized in opposite SCNs (n = 9; Fig. 2*B*) and differed in phase as well as in interval. Burst synchrony was maintained for 18.5 h (n = 2), and normal activity could be restored within 12 min in normal Ca^{2+} solution. Simultaneous single-unit activity and MUA recordings revealed that 61% of all neurons (n = 23) generated bursts and that most neurons (79% of the bursting neurons or 57% of all neurons) fired action potential(s) only during the MUA bursts. Intraburst interval and burst duration

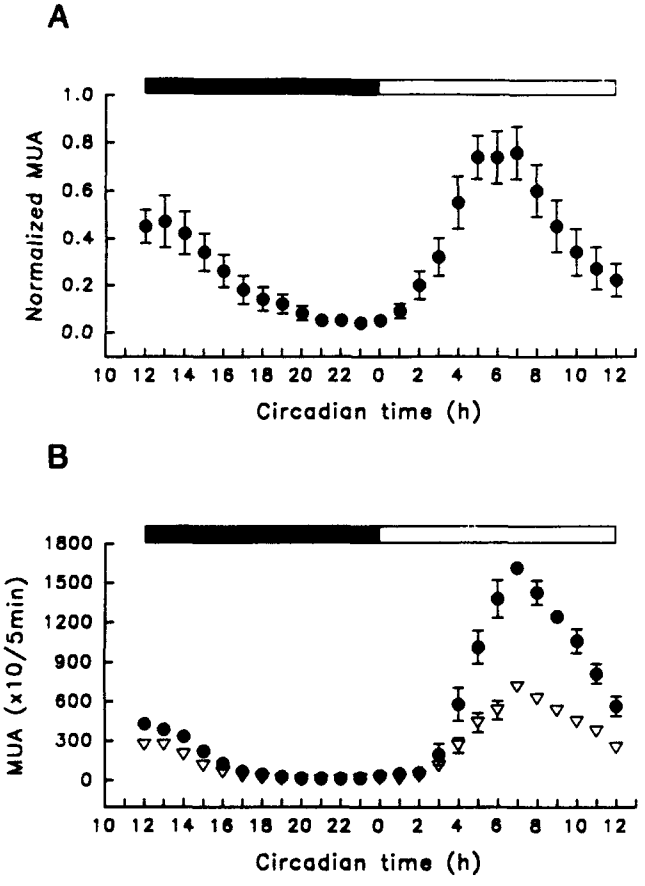

FIG. 1. Circadian rhythm of neuronal activity and comparison of activity recorded simultaneously from two locations in the SCN. (*A*) Average of normalized MUA (●) at each circadian hour from six rats. Normalized MUA was computed by dividing each hourly MUA count by the maximum hourly MUA count of each animal. (*B*) Averaged MUA count per 5 min at each circadian hour from one animal. MUA from the ventral SCN (●) and from 200 μm dorsal in the same SCN (▽) were counted simultaneously. Vertical lines are standard errors. Solid bars represent dark hours and open bars represent light hours.

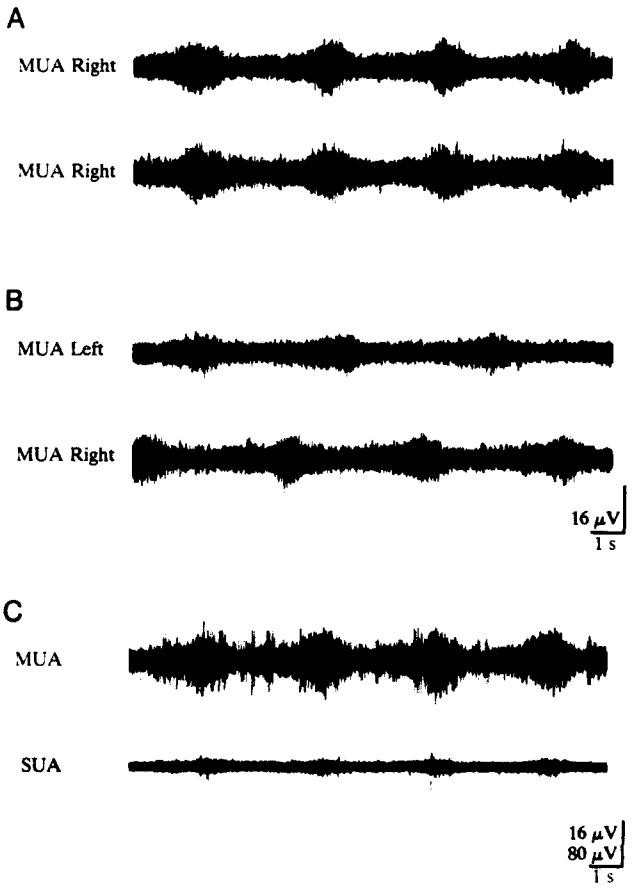

FIG. 2. Simultaneous extracellular recordings during bursting activity in Ca^{2+}-free solution. (*A*) Simultaneous recordings of MUA from two locations (150-μm separation) in the same SCN (right side) indicate synchronous bursts. The interburst interval gradually increased from 1 s to a maximum of up to 10 s after several hours, and the burst duration ranged from 0.5 to 2.0 s. (*B*) Simultaneous recordings of MUA from opposite SCNs (left and right) indicate that the bursts were not synchronous and had different interburst intervals (recorded in the same slice as in *A*). (*C*) Single-unit activity (SUA) recording during a MUA burst in the same SCN; the single-unit activity bursts coincided with the MUA bursts.

varied considerably between neurons and between consecutive bursts of the same neuron. Bursts of action potentials in individual cells occurred simultaneously with periodic increases of MUA amplitude (Fig. 2*C*), which demonstrates directly that MUA bursts represent action potentials from a population of bursting neurons.

Excitatory amino acid receptor antagonists and bicuculline block all evoked and spontaneous postsynaptic events in SCN neurons (12–15). Although unlikely, spontaneous Ca^{2+}-independent release of neurotransmitters (i.e., not mediated by action potentials) could conceivably contribute to burst synchronization. To test this hypothesis, we bath-applied glutamate (NMDA and non-NMDA) and $GABA_A$ receptor antagonists (100 μM 2-amino-5-phosphonopentanoic acid, 50 μM 6,7-dinitroquinoxaline-2,3-dione, and 50 μM bicuculline) in the Ca^{2+}-free solution. Although these antagonists led to a slight increase in interburst interval, they never altered MUA burst synchrony (n = 6; tested for 60–100 min), indicating that spontaneous release of excitatory and inhibitory amino acids was not required for synchronization. Intracellular recordings have revealed only *fast* PSPs mediated by amino acid receptors (i.e., NMDA, non-NMDA, and $GABA_A$ receptors) in the nucleus (refs. 12–14, 16, and 17; also see Fig. 3). Since all detectable PSPs were blocked, this result also rules out synchronization of SCN neurons through Ca^{2+}-independent transmitter release, which has previously been demonstrated in fish retinal neurons (18).

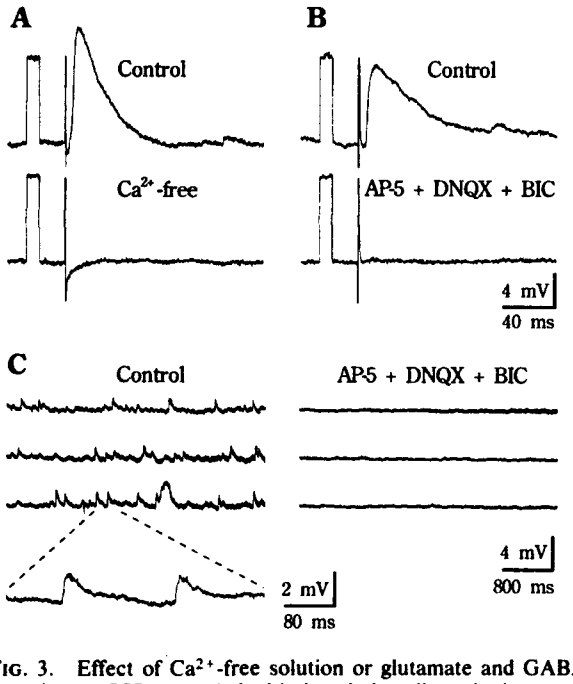

FIG. 3. Effect of Ca^{2+}-free solution or glutamate and $GABA_A$ antagonists on PSPs recorded with the whole-cell patch-clamp technique. (*A*) The upper trace shows an excitatory PSP evoked by extracellular stimulation dorsal to the SCN. The excitatory PSP was completely blocked (lower trace) after 17 min (range of 9–26 min; n = 4) in Ca^{2+}-free solution. Full recovery was obtained after 10 min in normal Ca^{2+} solution (not shown). The membrane potential in both traces was −66 mV. (*B*) The upper trace shows an evoked excitatory PSP from another neuron, which was blocked in a 100 μM 2-amino-5-phosphonopentanoic acid (AP-5), 50 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 50 μM bicuculline (BIC) mixture in normal Ca^{2+} (lower trace) after 13 min (range of 9–13 min; n = 3). The membrane potential in both traces was −78 mV. (*C*) The three upper left traces show continuous recordings of spontaneous PSPs in the same neuron as in *B*. The lower left trace shows two spontaneous PSPs at faster sweep speed and higher gain. The three right traces show a continuous recording during complete blockade of spontaneous PSPs after 12 min (range of 9–14 min; n = 6) in the antagonist mixture and normal Ca^{2+}. All traces in *C* were recorded at a membrane potential of −78 mV. Traces in *A* and *B* contain a calibration pulse (10 mV, 10 ms) before the truncated stimulus artifact.

To assess directly the effect of the Ca^{2+}-free solution and the receptor antagonists on chemical synaptic transmission in SCN neurons, we recorded spontaneous and evoked PSPs by using the whole-cell patch-clamp technique, which allows high resolution of synaptic events. Evoked excitatory PSPs were blocked in Ca^{2+}-free solution or during bath application of the amino acid antagonists (Fig. 3 *A* and *B*). Spontaneous PSPs were not blocked in the Ca^{2+}-free solution (n = 5; data not shown), but they were always abolished after application of the antagonists (Fig. 3*C*). Therefore, high-resolution whole-cell recordings directly demonstrated that all detectable chemical synaptic transmission was blocked in conditions that induced synchronized bursting when antagonists were present.

DISCUSSION

The synchronized bursts in Ca^{2+}-free solution represent a loose type of neuronal synchronization (i.e., many neurons firing within the same time window, but with variable discharge patterns and burst durations). To our knowledge, this type of Ca^{2+}-independent synchronization has not been described previously in the mammalian brain. Normal synchronization of SCN activity during the circadian rhythm (see Fig. 1*B*) has similarities to that seen in Ca^{2+}-free conditions, since at any given time a sample of neurons shows a wide range of firing frequencies (4–7) while still being loosely synchronized. The persistence of the synchronous bursts induced by Ca^{2+}-free solution, despite complete block of all detectable chemical synaptic transmission, demonstrates that a communication mechanism(s) other than Ca^{2+}-dependent synaptic transmission must operate in the SCN. This mechanism can synchronize neuronal activity within a nucleus but not between opposite nuclei (Fig. 2*B*). Communication between opposite nuclei is probably mediated by chemical synaptic transmission (16, 19) and therefore was blocked in the absence of Ca^{2+}.

Neurons in the inferior olive (20) and locus coeruleus (21) generate Ca^{2+}-dependent subthreshold oscillations that are rhythmic and synchronous. Although these oscillations are synchronized by gap junctions, and thus also seem to be independent of chemical synaptic mechanisms, they are Ca^{2+}-dependent and therefore different from the bursts described here. In hippocampal slices, low Ca^{2+} solutions produce synchronized bursts in the absence of active chemical synapses (22–24), but the action potentials are tightly synchronized and generate population spikes during these bursts. The SCN bursts differ from the hippocampal activity (i.e., weaker synchronization whereby action potentials do not occur one-for-one) and therefore represent a different type of synchronization of neuronal activity in the mammalian brain.

The neuronal synchronization in the hypothalamus that we are reporting here opens several additional lines of investigation. This Ca^{2+}-independent mechanism(s) of synchronization in the SCN may depend on electrotonic coupling via gap junctions, electrical field effects (ephaptic interactions), and/or changes in the concentration of extracellular ions (25). Among these three possible mechanisms, electrical field effects seem the most unlikely to operate in the SCN due to the lack of parallel arrangement of neuronal processes in the nucleus. Electrotonic coupling and ionic interactions are more likely to facilitate synchronization in the nucleus, since many SCN cell bodies are tightly packed together with extensive regions of membrane apposition between them (19). Although there is evidence for electrotonic coupling in some areas of the hypothalamus (26), nearly all of the data are derived from anatomical rather than electrophysiological

studies, and virtually no data are available for most hypothalamic areas including the SCN. It should now be possible to determine which hypothetical mechanisms of neuronal interaction are present in the SCN and the role they play in synchronization.

Previous indirect evidence has suggested that neurons in the SCN can be synchronized via nonsynaptic mechanisms. The circadian rhythm of glucose utilization that occurs across cells in the SCN is expressed before chemical synapses are functional (27). Tetrodotoxin application, which blocks synaptic transmission mediated by action potentials in axons, also blocks the circadian rhythm of motor activity while leaving the coordinated timing mechanism of the cellular clock in the SCN unimpaired (28). The nonsynaptic mechanism(s) demonstrated here could explain both observations, since neuronal activity could be synchronized by electrical and/or ionic interactions. A Ca^{2+}-independent mechanism(s) of neuronal synchronization may coordinate the cellular elements in the SCN responsible for circadian rhythms in mammals. Synchronization of neuronal activity in the SCN and other areas of the hypothalamus must be critical to execute the widespread behavioral, physiological, and endocrine effects. Nonsynaptic communication between mammalian neurons has not received the attention it deserves, but accumulating evidence (20–24, 29), as well as the present study, suggests that nonsynaptic mechanisms may have a major role in synchronizing neuronal activity in the mammalian brain.

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Patch-Clamp Analysis of Spontaneous Synaptic Currents in Supraoptic Neuroendocrine Cells of the Rat Hypothalamus

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Spontaneous synaptic currents were recorded in supraoptic magnocellular neurosecretory cells using whole-cell patch-clamp techniques in the rat hypothalamic slice preparation. Numerous spontaneous excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were observed in the 27 cells recorded. The rate of occurrence of the spontaneous currents and the relative proportion of EPSCs versus IPSCs varied significantly from cell to cell. Miniature EPSCs and IPSCs were clearly distinguished from background noise in TTX ($n = 3$ cells at 0.5 μg/ml). The frequency of EPSCs and IPSCs decreased by approximately 70% and the largest events were blocked in TTX, but the peaks of the amplitude histograms were shifted by only a few picoamperes. Bicuculline ($n = 10$ cells at 10 μM and 2 cells at 20 μM) blocked completely all the IPSCs without any detectable effect on the frequency or amplitude of the EPSCs. No slow spontaneous outward currents, indicative of a K^+ current from activation of $GABA_B$ receptors, were observed. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate-type glutamate receptor antagonist 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX; $n = 7$ cells at 10 μM) consistently blocked all EPSCs without any apparent effect on the frequency or amplitude of the IPSCs. No synaptic events could be detected when CNQX was applied in combination with bicuculline ($n = 4$). The decay phase of averaged spontaneous IPSCs and EPSCs recorded at resting membrane potential could be well fitted by single exponential functions in most cells. The time constants ranged from 0.92 to 3.0 msec for EPSCs (five cells) and from 5.3 to 6.6 msec for IPSCs (four cells). A second, slower time constant of 4–15 msec was found in the largest averaged EPSCs (≥40 pA). The amplitude of this slow component was −2 to −4 pA. These results suggest that, in the *in vitro* slice preparation, glutamate mediates all the spontaneous EPSCs in magnocellular neurosecretory cells by acting primarily on AMPA/kainate-type receptors at resting membrane potential and that activation of $GABA_A$ receptors mediates most if not all IPSCs.

[Key words: hypothalamus, supraoptic nucleus, neurosecretion, magnocellular neuroendocrine cells, amino acids, synaptic currents, patch clamp]

Magnocellular neuroendocrine cells of the supraoptic nucleus have long been considered as a model system for a wide range of studies on neurosecretion. These neuroendocrine cells synthesize the neuropeptide hormones oxytocin and vasopressin and transport them, from their cell bodies in the supraoptic and paraventricular nuclei, along their axons to the neurohypophysis where they are secreted directly in the general circulation. The supraoptic nucleus has particular advantages for electrophysiological studies on neuroendocrine cells because, unlike other hypothalamic nuclei, virtually all of these cells project to the neurohyphophysis and are therefore neuroendocrine. Numerous substances (e.g., ACh, norepinephrine, opioid peptides, dopamine, histamine, 5-HT, substance P, vasopressin, oxytocin, cholecystokinin, somatostatin) have been proposed to regulate or modulate hypothalamic neurosecretion (for review of the neuropharmacology of this system, see Renaud and Bourque, 1991). A wide range of studies on conventional neurons, as opposed to hypothalamic neuroendocrine cells, suggest that these substances primarily serve as neuromodulators rather than neurotransmitters responsible of traditional synaptic potentials. Until recently, however, relatively few studies have directly tested the hypothesis that excitatory and inhibitory amino acids (i.e., glutamate and GABA) mediate all fast synaptic transmission in the magnocellular neuroendocrine system (Gribkoff and Dudek, 1988, 1990; Wuarin and Dudek, 1991). Recent evidence suggests that most of the excitatory and inhibitory synapses, not only in the magnocellular system, but throughout the neuroendocrine hypothalamus may be glutamatergic and GABAergic, respectively (van den Pol et al., 1990).

Studies using intracellular recordings showed that two broad-spectrum antagonists for excitatory amino acids, kynurenic acid and γ-*d*-glutamylglycine, inhibit electrically evoked and spontaneous EPSPs in the rat supraoptic nucleus (Gribkoff and Dudek, 1988, 1990). Most of the electrically induced excitatory synaptic input to magnocellular and parvocellular neurons in the paraventricular nucleus and to neurons in the arcuate nucleus has been shown to be inhibited or blocked by 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), suggesting that glutamate mediates excitatory synaptic events mainly by activating AMPA/kainate-type receptors (van den Pol et al., 1990; Wuarin and Dudek, 1991). Immunohistochemical studies have shown glutamate-reactive presynaptic boutons in contact with dendrites and cells bodies in the supraoptic nucleus (Meeker et al., 1989; van den Pol et al., 1990). Therefore, recent electrophysiological and immunohistochemical observations have provided evidence that glutamate is a neurotransmitter at many excitatory synapses in the supraoptic nucleus and elsewhere in the hypothalamus.

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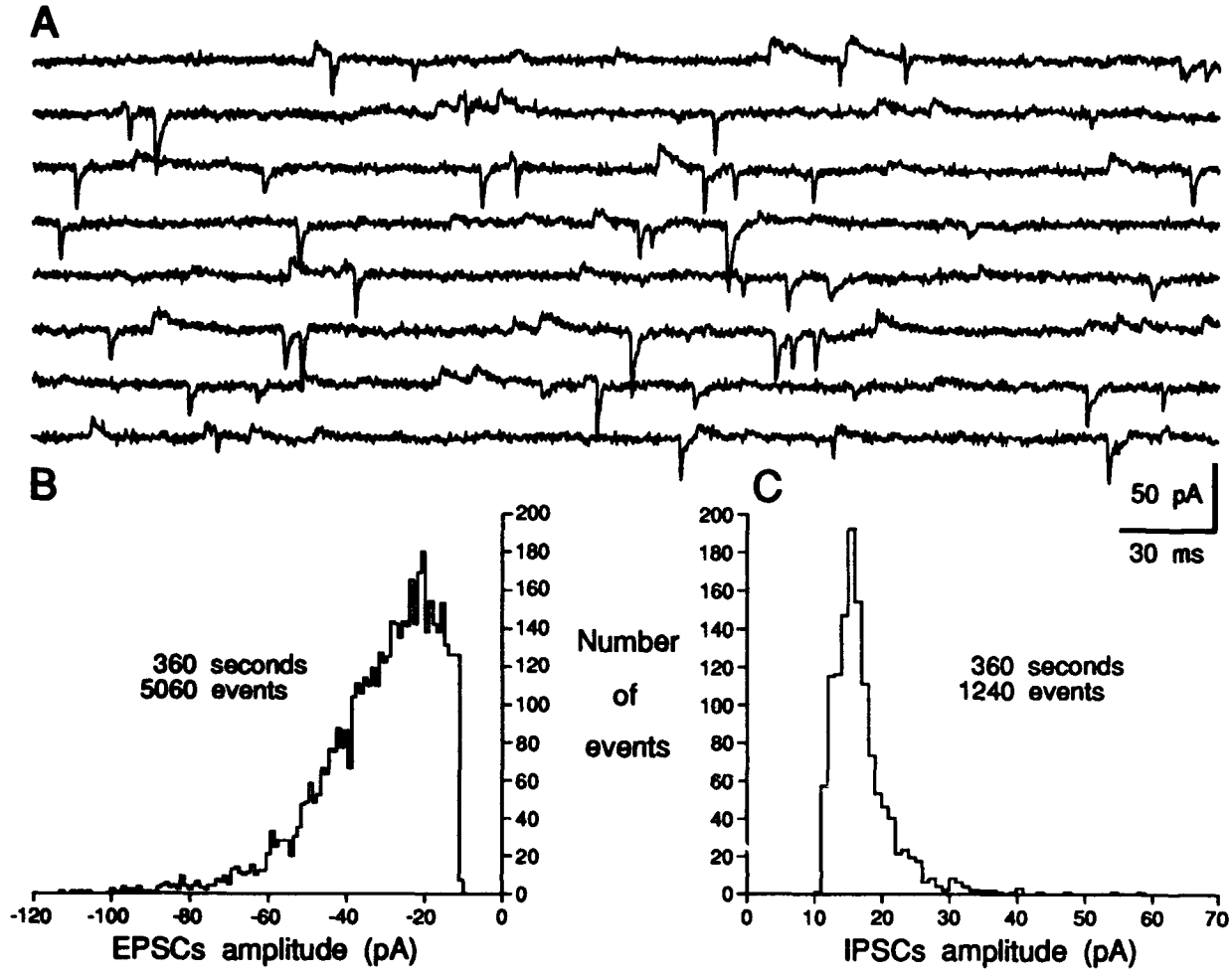

Figure 1. Whole-cell patch-clamp recording of a supraoptic magnocellular neuroendocrine cell with both excitatory and inhibitory inputs. *A–C* are data from the same cell. *A*, Spontaneous currents recorded at resting membrane potential (−66 mV); R_{in} was 700 MΩ. Traces are continuous. *B* and *C*, Histograms showing distribution of the EPSC and IPSC amplitudes, respectively. Current amplitudes were measured during 6 min epochs from data such as shown in *A*. Note that in this cell there were approximately four times more EPSCs than IPSCs.

A recent anatomical study using post-embedding surface staining with immunogold showed that GABA-immunoreactive terminals compose approximately 50% of the synaptic input, not only to the supraoptic nucleus, but also to paraventricular, arcuate, and suprachiasmatic nuclei. This observation strongly suggests that GABA is the most important inhibitory neurotransmitter in the magnocellular system and in the hypothalamus (Decavel and van den Pol, 1990). Intracellular recordings from supraoptic neurons obtained in the explant preparation showed an abundance of spontaneous IPSPs that were chloride dependent and mediated by $GABA_A$ receptor activation (Randle et al., 1986). Extracellular stimulation in the diagonal band of Broca evoked IPSPs that closely resembled the spontaneous events. These results suggest that supraoptic magnocellular neurons receive an extensive and powerful inhibitory GABAergic input, originating at least in part in structures located rostral to the supraoptic nucleus.

The electrophysiological studies mentioned above used sharp microelectrodes for intracellular recording. This technique allows detection of spontaneous EPSPs but has limitations regarding the resolution of small events. Synaptic potentials mediated by substances other than glutamate or GABA may be relatively small and may not have been detected. Another potential problem of these previous studies is the use of extracellular electrical stimulation to evoke synaptic responses. The stimuli were delivered through electrodes positioned near the supraoptic or paraventricular nucleus, a location that gave large and consistent synaptic responses. With this method, however, it is likely that only a fraction of the entire input to a nucleus is activated, and that afferent fibers releasing neurotransmitters other than amino acids were not stimulated. In the present study, we used whole-cell patch-clamp recordings in conventional hypothalamic slices (Blanton et al., 1989) to address the two problems mentioned above. This technique provides a signal-to-noise ratio far superior to intracellular recording with sharp electrodes and allows detection of miniature synaptic events (Edwards et al., 1990; Otis et al., 1991). Testing the effects of amino acid receptor antagonists on spontaneous EPSCs and IPSCs would be expected to provide a more rigorous assessment of the importance of the excitatory and inhibitory amino acids (i.e., glutamate and GABA) as transmitters for the neuroendocrine cells in the supraoptic nucleus.

The aims of this study were (1) to establish the properties of spontaneous postsynaptic currents in supraoptic magnocellular neuroendocrine cells; (2) to determine if glutamate, acting on AMPA/kainate-type receptors, mediates all detectable excitato-

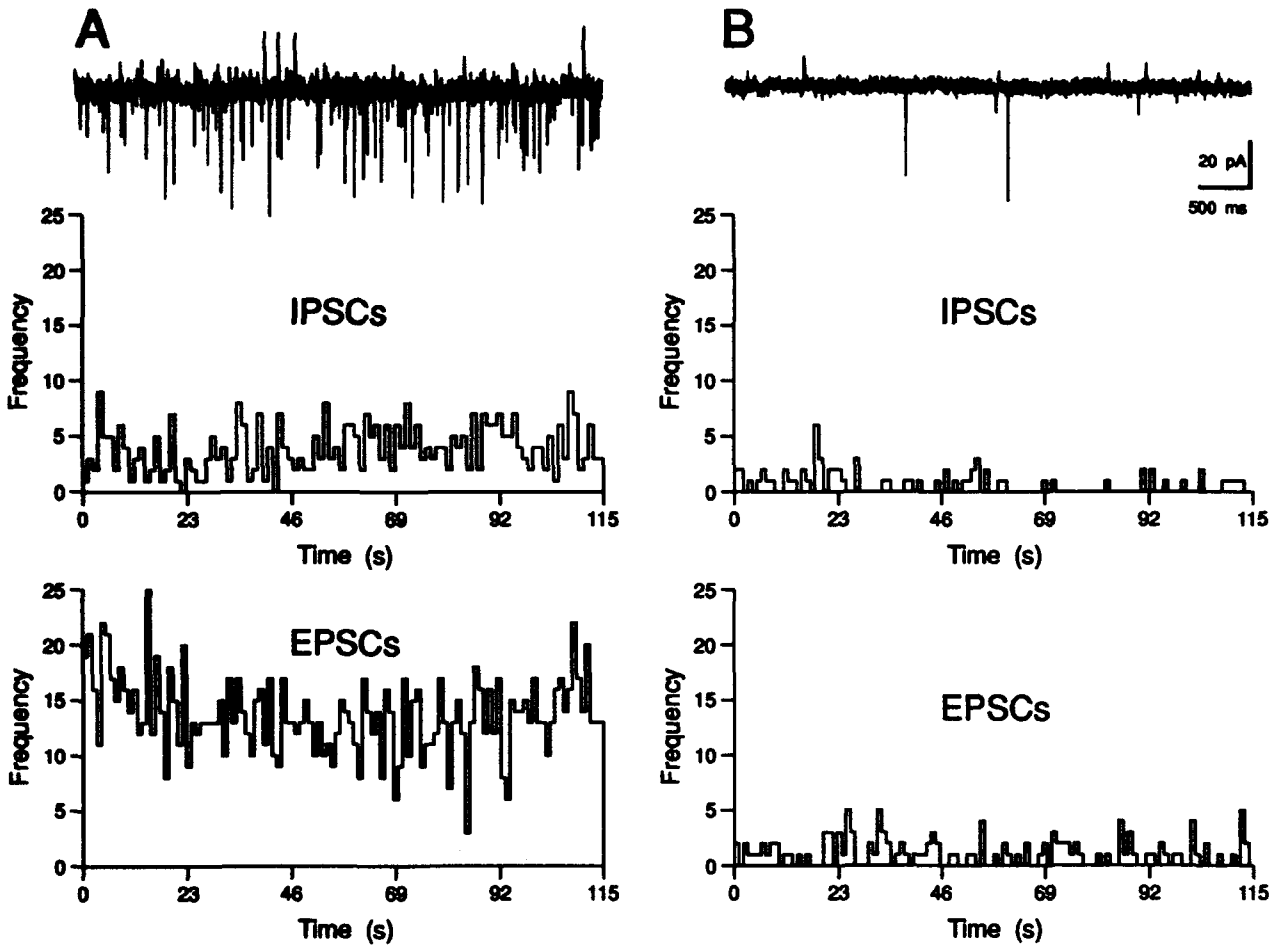

Figure 2. Comparison of the rate of spontaneously occurring synaptic events in two different cells. Traces in *A* and *B* show two samples (5 sec) of spontaneous synaptic activity at a relatively slow time scale. Upward deflections were IPSCs and downward defections were EPSCs. Frequency histograms of IPSCs and EPSCs (during 115 sec) are shown for each cell. The average rate for IPSCs and EPSCs was, respectively, 4.2 sec^{-1} and 13 sec^{-1} for the cell in *A* and 0.7 sec^{-1} and 1.1 sec^{-1} for the cell in *B*.

ry synaptic currents; (3) to test if $GABA_A$ receptors mediate all detectable IPSCs; and (4) to determine if activation of NMDA receptors contributes to the decay of EPSCs. The properties and components of the EPSCs and IPSCs were examined through analyses of the time course of the events, and the role of AMPA/kainate and $GABA_A$ receptors was evaluated with bath application of CNQX and bicuculline.

Materials and Methods

Slice preparation. Immature male rats (50–80 gm) were decapitated, and their brains rapidly dissected and placed in ice-cold perfusion solution (see below) for 1 min. During the dissection, particular care was taken to cut the optic nerves without applying any tension on them. A block of tissue containing the hypothalamus was then trimmed and glued on the stage of a vibrating microtome. Usually two slices of 400–600 μm containing the supraoptic nucleus were cut in a frontal plan and immediately transferred in a ramp chamber (Haas et al., 1979) oxygenated with 95% O_2, 5% CO_2 and thermoregulated at 34°C. The perfusion solution contained (in mM) 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.3 $MgSO_4$, 1.4 NaH_2PO_4, 2.4 $CaCl_2$, and 10 glucose; pH 7.4. The following drugs were applied in the perfusion solution: bicuculline methiodide (10 and 20 μM), TTX (0.5 μg/ml) (both from Sigma, St. Louis, MO), and CNQX (3 and 10 μM, from Tocris Neuramin, Buckhurst Hill, England).

Recording methods. Whole-cell current recordings (Hamill et al., 1981) were obtained using patch pipettes with open resistances of 2–5 MΩ. Seal resistances were 1–10 GΩ; series resistances were 5–25 MΩ compensated at >80%. Patch pipettes were filled with (in mM) 140 K-gluconate, 10 HEPES, 1 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 BAPTA, and 4 ATP (pH 7.2). ATP was used to decrease the rundown of IPSCs. Without 4 mM ATP, IPSCs gradually disappeared within the first 20 min (Stelzer et al., 1988). The pipettes were pulled from borosilicate glass capillaries of 1.7 mm diameter and 0.5 mm wall thickness. Currents were recorded using an Axopatch-1D amplifier, low-pass filtered at 2 kHz, digitized at 44 kHz (Neuro-Corder, Neurodata Inc.), and stored on videotapes. The amplitude and decay phases of spontaneous synaptic currents were measured on a personal computer using PCLAMP programs (Axon Instruments) with a sampling rate of 10 kHz. Spontaneous currents were recorded at resting membrane potential, which was less negative than the reversal potential for IPSPs (Randle et al. 1986); therefore, inward currents were considered as EPSCs and outward currents as IPSCs.

Cell labeling. Six cells were recorded with pipettes containing biocytin (0.2%, $n = 2$; 0.4%, $n = 4$). At the end of the experiment, the slice containing the marked cell was immersed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer overnight. The tissue was then cut at 50 μm and rehydrated. The sections were labeled with an avidin–biotinylated horseradish peroxidase reaction (Horikawa and Armstrong, 1988). To ascertain that the labeled cells were within the boundaries of the supraoptic nucleus, the slices were counterstained with cresyl violet.

Results

Recordings were obtained from 27 cells in 23 slices. Once the whole-cell configuration was established, stable recordings were achieved for several hours without noticeable change in resting

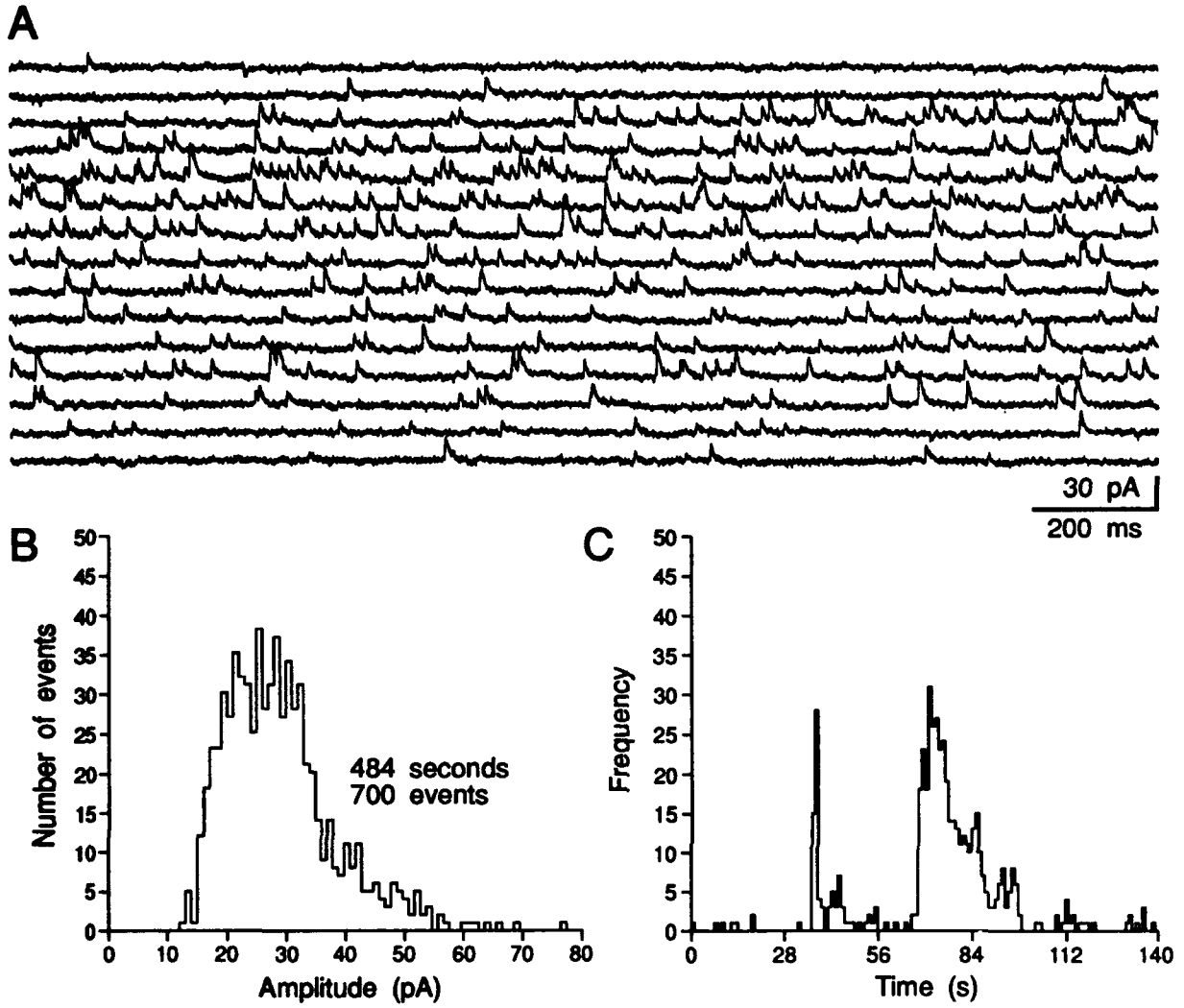

Figure 3. Whole-cell recording of a magnocellular neuron showing predominantly IPSCs. This cell showed bursts of IPSCs separated by periods of relative inactivity. *A*, Example of a burst of IPSCs. Between bursts, the presynaptic cell was presumably silent (see *top* and *bottom two traces*). Traces are continuous. *B*, Amplitude histogram of IPSCs from cell shown in *A* measured during a period of approximately 8 min. *C*, Frequency of spontaneous IPSCs during a period of 2 min 20 sec, including two bursts of IPSCs. The second peak corresponds to the burst shown in *A*. Recording was at resting membrane potential (−60 mV); R_{in} was 900 MΩ.

membrane potential or input resistance. Mean resting membrane potential was −63 ± 2.5 mV (mean ± SE, n = 21), and input resistances ranged from 400 to 1100 MΩ with a mean of 726 ± 50 MΩ (mean ± SE, n = 25). All recordings were made with the holding potential set to the same value as the resting membrane potential for the recorded cell.

Although it was relatively easy to position recording electrodes within the supraoptic nucleus under visual control using slices cut in the frontal plan, we also recorded from six cells with pipettes containing biocytin in order to label them and verify that we were indeed recording from magnocellular neurons. This confirmed that we were not recording from cells at the periphery of the nucleus that have been hypothesized to be GABAergic neurons (Theodosis et al., 1986). We recovered four cells, and they were located well within the boundaries of the supraoptic nucleus (two cells could not be found in the stained sections).

Numerous spontaneous synaptic events were observed in all cells. Inward currents were defined as EPSCs and outward currents as IPSCs (Fig. 1). The IPSC amplitudes ranged from 10 to 80 pA, and the average amplitude for each cell was between 15 and 30 pA. The EPSC amplitudes ranged from −10 to more than −150 pA, and the average amplitude was from −10 to −50 pA. The rate of occurrence of spontaneous EPSCs and IPSCs varied from <1 Hz to >10 Hz (Fig. 2). Three cells showed bursts of spontaneous IPSCs, and the maximum frequency of IPSCs during a burst was >20 Hz (Fig. 3). The proportion of EPSCs versus IPSCs also varied from cell to cell; four cells showed mostly IPSCs, and two cells showed only EPSCs.

Effects of TTX on synaptic currents

We applied TTX in the perfusion solution at 0.5 μg/ml to test if synaptic activity could be detected after action potentials had been blocked (Fig. 4). In the three cells tested, miniature EPSCs and IPSCs could be clearly distinguished from baseline noise. Figure 5 shows an example of amplitude distributions of spontaneous EPSCs and IPSCs before and during the application of TTX.

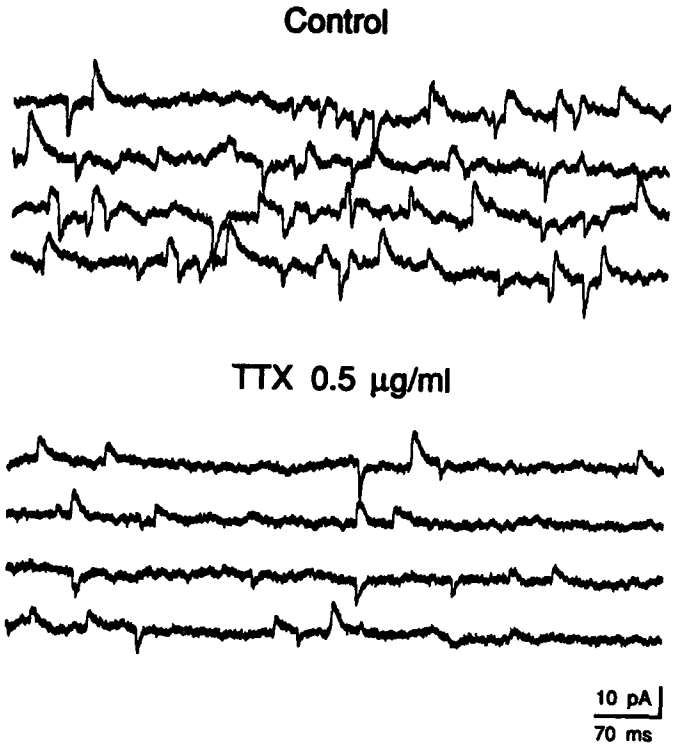

Figure 4. Comparison of spontaneous synaptic activity in normal perfusion solution (*top*) and 20 min after addition of 0.5 μg/ml TTX (*bottom*). The cell R_{in} was 500 MΩ. Traces are continuous, and holding potential was the same as resting membrane potential (−55 mV).

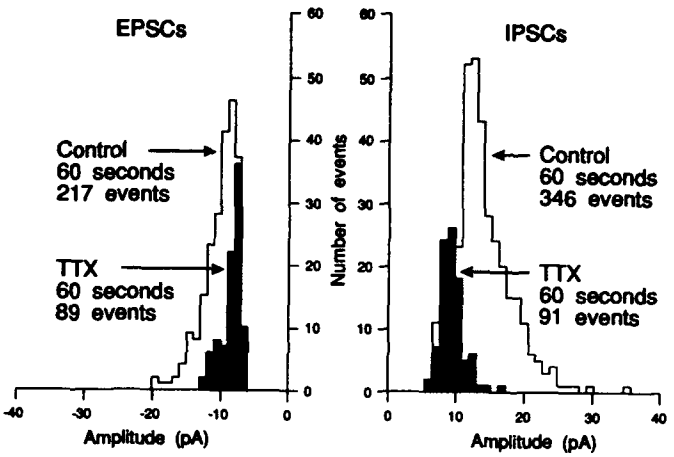

Figure 5. Histograms of the amplitudes of EPSCs (*left*) and IPSCs (*right*) recorded from the cell shown in Figure 4 in normal solution and in TTX-containing solution. In each condition, EPSCs and IPSCs were measured during a 1 min period. The *solid columns* represent the amplitudes of EPSCs and IPSCs recorded in the presence of 0.5 μg/ml TTX. Note that the peak of the EPSC amplitude histogram was decreased only from −9 to −8 pA by TTX. The peak of the IPSC amplitude distribution was decreased from −13 to −10 pA.

Effect of bicuculline and CNQX on spontaneous synaptic currents

To determine if all the IPSCs were due to the activation of $GABA_A$ receptors, we tested bicuculline on 10 cells at 10 μM and on 2 cells at 20 μM. Bicuculline, at both concentrations, blocked completely all the IPSCs in all the cells tested. This block was reversible (Fig. 6). Bicuculline had no effect on the frequency and amplitude of EPSCs and it did not modify input resistance or resting membrane potential.

In order to test the hypothesis that all spontaneous EPSCs in magnocellular neuroendocrine cells are mediated by glutamate, we applied the non-NMDA receptor-selective antagonist CNQX in the perfusion solution. The antagonist was tested on six cells at 10 μM and on one cell at 3 μM. In the seven cells tested, CNQX reversibly blocked all the EPSCs. In three cells, bicuculline (20 μM) was applied before CNQX to block IPSCs and isolate the EPSCs. When CNQX was then added to bicuculline in the perfusion solution, no synaptic activity could be detected (Fig. 7).

Time course of decay phase of spontaneous currents

Decays of spontaneous currents were measured from averaged EPSCs and IPSCs recorded at resting membrane potential. The synaptic currents were averaged by using the first half of the rising phase to align them. Both EPSCs and IPSCs could be fitted by a single exponential function (Fig. 8). The fit was quite good for all the averaged IPSCs. Decays of the smaller averaged EPSCs (i.e., ≤30 pA) could be well fitted by a single exponential (τ = 2.4 ± 0.38 msec; n = 4; e.g., Fig. 8, cells 1, 3, and 4). However, in four of the larger averaged EPSCs (≥40 pA; e.g., Fig. 8, cells 2 and 5), the decay was better fitted by the sum of two exponentials. The second time constant second was 4–15 msec, and its amplitude was −2 to −4 pA. No significant difference was found in decay time constant of averages of the smallest and the largest synaptic currents (Fig. 9).

Discussion

Patch-clamp recordings of spontaneous currents versus previous studies

The present study used whole-cell patch-clamp recordings in the "thick slice" (Blanton et al., 1989) to investigate the electrophysiology of the magnocellular system. All the previous studies of neurotransmission in the magnocellular system used sharp microelectrodes, which provide relatively low signal-to-noise ratio. The necessity of using particularly high-resistance electrodes to obtain stable recordings in magnocellular neuroendocrine cells compounded the problem of the low resolution of this type of electrode and made it difficult to record spontaneous events routinely. Only high-quality recordings allowed clear detection of spontaneous synaptic activity (Randle et al., 1986; Gribkoff and Dudek, 1990). The previous work was also based mostly on the study of electrically evoked synaptic responses. The rationale for this approach was to obtain responses that were large enough for accurate measurement of the effects of selective antagonists. After averaging, this allowed analysis of dose–response relations (Wuarin and Dudek, 1991). One of the weaknesses of the electrical stimulation technique is that one can only activate a particular pathway(s), and therefore evoked synaptic responses may not reveal all of the afferent inputs to a cell. Considering these limitations and the amount of evidence accumulated over the years in favor of other neurotransmitters in the magnocellular system, we reexamined the importance of glutamatergic and GABAergic neurotransmission in the magnocellular system by studying spontaneous synaptic events with a technique that allows greatly superior resolution over sharp microelectrodes.

The improved signal-to-noise ratio provided by patch pipettes allowed us to detect spontaneous miniature synaptic currents

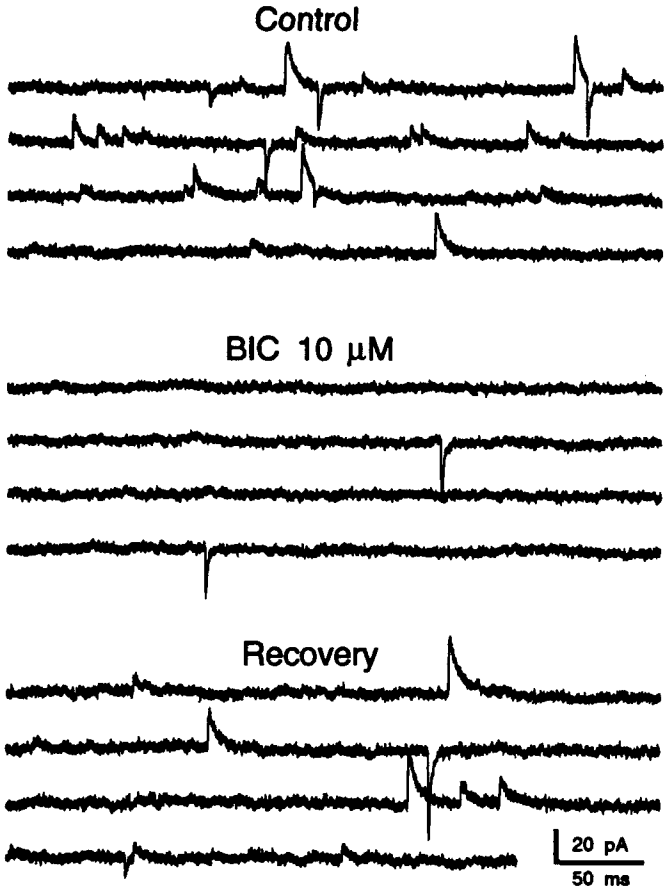

Figure 6. In bicuculline, no IPSCs were detected. *Upper panel* shows recording under control conditions. *Middle panel* shows the effect of bath-applied bicuculline (15 min at 10 μM). The IPSCs recovered from the block, but only after more than 90 min of washout (*bottom panel*). All traces are continuous, holding potential was −70 mV throughout the experiment, and cell R_{in} was 1 GΩ.

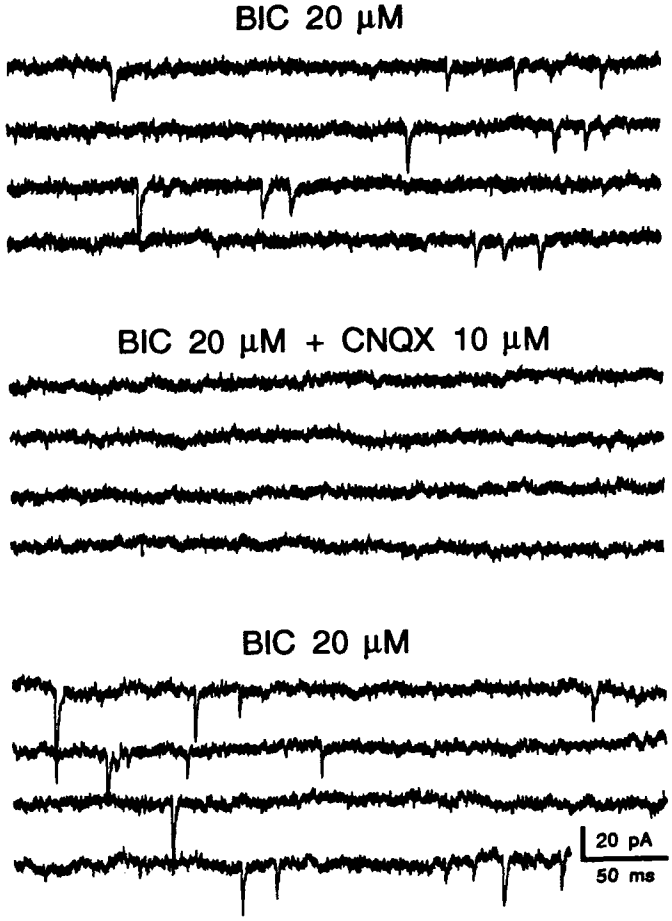

Figure 7. No spontaneous EPSCs or IPSCs were detected in the presence of bicuculline and CNQX. As shown in Figure 6, no IPSCs were observed in bicuculline (20 μM; *upper panel*). When CNQX (10 μM) was added to the perfusion solution in the presence of bicuculline (20 μM), all spontaneous synaptic currents were blocked (*middle panel*). The effect of CNQX could be reversed after approximately 120 min (*bottom panel*). Cell R_{in} was 600 MΩ; holding potential was resting membrane potential (−65 mV). Traces are continuous.

in all the cells tested with TTX. The amplitude of the miniature EPSCs and IPSCs was well above noise level in each cell tested. It is therefore reasonable to assume that we would have been able to detect any spontaneous EPSCs and IPSCS not mediated by glutamate or the activation of $GABA_A$ receptors when the non-NMDA receptor antagonist CNQX and bicuculline were present in the perfusion solution.

It has been shown that spontaneous GABA- and glutamate-mediated synaptic currents can be observed in an isolated mammalian central neuron (Drewe et al., 1988). These currents were Ca^{2+} dependent and blocked by γ-*d*-glutamylglycine and bicuculline. These results suggest that the presence of functional synaptic boutons attached to the postsynaptic cell is a sufficient condition for miniature spontaneous activity to occur, and that the cell bodies and the axons of the projecting cells are not necessary. Therefore, spontaneous activity in the slice may provide a qualitative means to study all of the inputs to a given cell.

One of the major limitations of the patch-clamp technique is the dialysis of the cell cytoplasm. The consequence is "run-down," which affects mainly currents dependent on the activation of G-protein–coupled receptors. Ligand-gated channels generating fast events, such as the nicotinic ACh receptor, seem to be less susceptible to washout. We did not detect any obvious decrease in the spontaneous activity within the first 3 min of recording, which suggests that rapid washout of synaptic currents after establishment of the whole-cell configuration did not occur. However, we found that Mg^{2+} in the intracellular solution was necessary to avoid washout of all IPSCs within 30 min. When Mg^{2+} was present in the pipette, we did not notice any obvious decrease in the frequency of spontaneous events, even over a period of several hours of recording. The fact that EPSCs and IPSCs recovered from the effect of CNQX and bicuculline, respectively, argues against the possibility that washout occurred during application of the antagonists.

EPSCs and glutamate

The results obtained in the present study support and extend the earlier work showing that kynurenic acid, γ-*d*-glutamylglycine, and CNQX inhibit evoked EPSPs in supraoptic and paraventricular magnocellular neuroendocrine cells (Gribkoff and Dudek, 1988, 1990; Wuarin and Dudek, 1991). As mentioned above, these studies used extracellular electrical stimulation to evoke synaptic responses and high-resistance sharp microelectrodes with relatively low resolution. Although these results

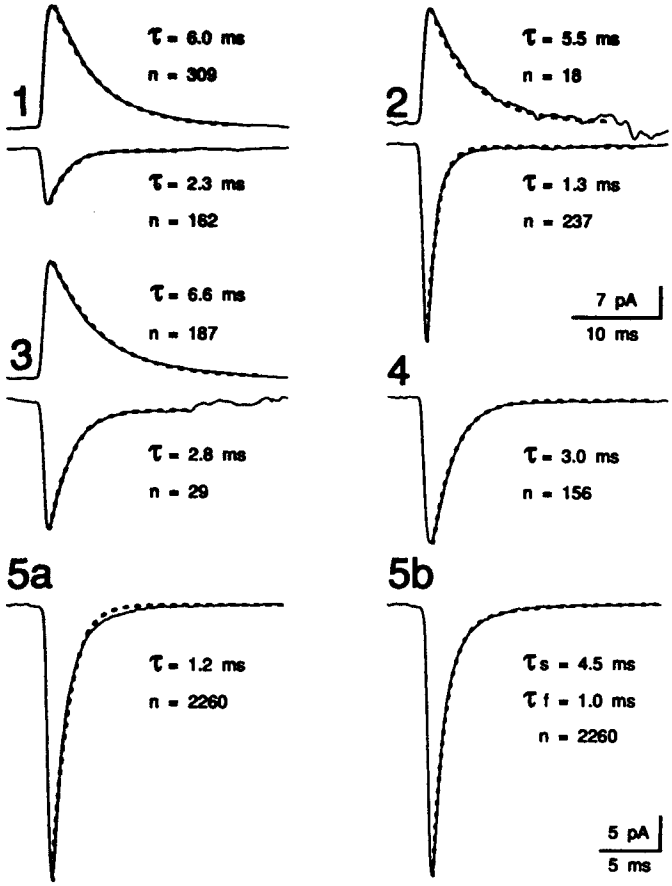

Figure 8. Decay phase of IPSCs and of most EPSCs was well fitted by single exponential functions. Traces are averaged spontaneous EPSCs and IPSCs from five different cells (*1–5*) obtained at resting membrane potential in normal solution. *Cell 4* did not show any IPSCs. The time constant of the fit (*broken line*) is indicated for each averaged current. The number of averaged events is shown by *n*. *Cell 5* is the same cell as shown in Figure 2*A*; however, only EPSCs were averaged. Traces in *5a* and *5b* are identical (note different calibration for *cells 1–4* and *cell 5*). The decay phase of *cell 5* was poorly fitted with a single exponential function (*5a*). The sum of two exponential functions (*5b*) shows a small component (−4 pA) with a relatively slow time constant (τs) and a larger component (−34 pA) with a fast time constant (τf).

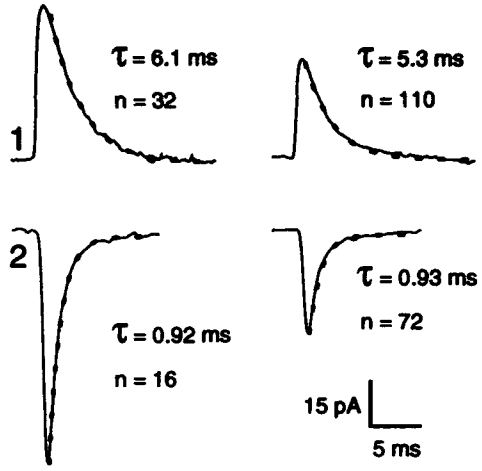

Figure 9. EPSCs and IPSCs of different amplitudes had similar decay phase time constants. Traces are averages of spontaneous IPSCs (*top traces, cell 1*) and EPSCs (*bottom traces, cell 2*) recorded at resting membrane potential in the control perfusion solution. The *broken lines* superimposed on each trace represent the fit of the decay phase of the synaptic current to a single exponential function. For each averaged current, time constant of the fit of the decay phase is indicated. The number of events per averaged trace is indicated by *n*. For each cell, averages of *n* of the largest and of the smallest postsynaptic currents are shown on the *left* and *right columns*, respectively. Spontaneous IPSCs (*cell 1*) were from the same cell as shown in Figure 3. *Cell 2* had a resting membrane potential of −58 mV, and its input resistance was 700 MΩ.

showed that glutamate is the major excitatory neurotransmitter in the magnocellular neuroendocrine system, the methods used did not allow one to exclude an important role of other neurotransmitters. There is an abundance of data showing that other neuroactive substances influence vasopressin and oxytocin secretion as well as increase the firing activity of magnocellular neurons. Among the most studied potential neurotransmitters with excitatory actions in the magnocellular system are ACh, norepinephrine, dopamine, histamine, cholecystokinin, and angiotensin (Renaud and Bourque, 1991). In other systems in the brain, these substances have been shown to have slow effects. In the magnocellular system, evidence has been presented suggesting the existence of a monosynaptic cholinergic input onto supraoptic neurons (Hatton et al., 1983). As described above, the signal-to-noise ratio obtained with patch pipettes would have allowed us to detect any small events mediated by neurotransmitters such as ACh or histamine (Yang and Hatton, 1989). We did not detect any inward current in the seven cells tested with CNQX. The present results support the hypothesis that glutamate probably mediates all the fast EPSCs in magnocellular neuroendocrine cells.

NMDA receptors and EPSCs

Several recent studies have demonstrated that NMDA receptors are present in supraoptic cells. A DL-2-amino-5-phosphonopentanoic acid (AP-5)-sensitive component to electrically evoked postsynaptic potentials could be revealed in the slice preparation when Mg^{2+} was deleted from the perfusion solution (Gribkoff, 1991). The amplitude of excitatory synaptic potentials evoked by electrical stimulation of the organum vasculosum lamina terminalis was increased in Mg^{2+}-free medium and inhibited by AP-5 (Yang and Renaud, 1991). Depolarization (Hu and Bourque, 1991a) and rhythmic bursting activity (Bourque and Hu, 1991; Hu and Bourque, 1991b) could be induced in magnocellular neurons by application of NMDA. These results suggest that NMDA receptors are present on these cells, that they influence their firing properties, and that they play a role in synaptic transmission.

In hippocampal granule cells (Keller et al., 1991), NMDA-dependent evoked EPSCs recorded in cells voltage clamped near resting membrane potential had a decay that could be fitted by a double exponential whose time constants were approximately 1 and 2 orders of magnitude slower than that of the non-NMDA component. In the thin cerebellar slice, evoked EPSCs in granule neurons had a slow component that could be blocked by AP-5. Miniature EPSCs were composed of an initial fast non-NMDA component followed by clearly discernable NMDA channels (Silver et al., 1992). Averaging of these miniature currents revealed that the channel openings formed a second slower synaptic component. The amplitude of the slow NMDA component, in both spontaneous and induced EPSCs, was approximately 10% of the fast component amplitude. To detect

a potential NMDA component in the spontaneous EPSCs at resting membrane potential, we measured the time course of the decay phase using exponential functions. We found that the decay phase of averaged spontaneous EPSCs in magnocellular neuroendocrine cells could be well fitted with one exponential in most cells. In a few cells, however, we found a second component characterized by a small amplitude (approximately 10% that of the fast component) and a time constant 5–10 times slower than the fast time constant. This slow phase of the decay of spontaneous EPSCs is reminiscent of an NMDA component, such as described in the studies mentioned above. Since most of the channels linked to the NMDA receptors are blocked at resting membrane potential by the Mg^{2+} present in the extracellular medium (Mayer et al., 1984; Nowak et al., 1984; Jahr and Stevens, 1990), NMDA currents are expected to be relatively small at that potential. Possible space-clamp problems might have allowed the largest EPSCs to depolarize the membrane potential sufficiently to allow NMDA receptors to be activated; however, since no significant difference was found in decay time constant of averages of the smallest and the largest EPSCs, the decay of the EPSCs was probably not determined by cell membrane passive properties (Llano et al., 1991). Taken together with results obtained using extracellular electrical stimulation in the paraventricular nucleus (Wuarin and Dudek, 1991), our results do not support the notion of a significant activation of NMDA receptors in spontaneous EPSCs of supraoptic magnocellular neurons at resting membrane potential. This contrasts with results obtained in cerebellar granule cells (Silver et al., 1992), hippocampal granule cells (Keller et al., 1991), and neocortical pyramidal cells (Wuarin et al., 1992) where a more consistent NMDA component to the excitatory synaptic responses was found. Future experiments designed to evaluate the voltage dependence of spontaneous EPSCs are needed for a more complete evaluation of the role played by the NMDA receptors in synaptic transmission in the magnocellular neuroendocrine system.

GABA and IPSCs

GABA is considered to be the dominant inhibitory neurotransmitter in the magnocellular system (van den Pol, 1985; Jhamandas and Renaud, 1986; Randle et al., 1986; Renaud and Bourque, 1991) and throughout the hypothalamus (Decavel and van den Pol, 1990). However, other neuroactive substances have been shown to inhibit vasopressin and oxytocin secretion and to decrease the firing rate of magnocellular neuroendocrine cells. Examples are norepinephrine (Armstrong et al., 1982; Arnaud et al., 1983) and opioid peptides (Wuarin and Dudek, 1990). Using the same strategy that we used for the EPSCs, we searched for small or relatively slow events, once all the fast $GABA_A$ receptor–mediated currents were blocked by bicuculline. We did not detect any outward currents in the 12 cells tested with the $GABA_A$ receptor antagonist bicuculline. To our knowledge, there is no report of spontaneous $GABA_B$ receptor–mediated synaptic currents. Although it is possible that these currents could have been washed out immediately after obtaining the whole-cell recording configuration, no studies using sharp electrodes have presented evidence for spontaneous inhibitory synaptic events mediated by $GABA_B$ receptors. These results support the hypothesis that all IPSCs, at least all fast IPSCs, are mediated by activation of $GABA_A$ receptors.

The time constants of spontaneous IPSCs were faster than previously reported for spontaneous IPSPs measured in the hypothalamic explant preparation (Randle et al., 1986). This previous study used sharp electrodes and current-clamp recordings, and the decay of spontaneous IPSPs was measured on recordings done with microelectrodes filled with 3 M KCl. The longer time constant of the decay of IPSPs reflects the passive properties of the membrane, which are classically considered to set the slow decay of IPSPs.

Source of spontaneous currents

Studying spontaneous synaptic currents has advantages over electrically evoked responses for assessing the overall synaptic input to a cell. In the *in vitro* slice preparation, however, several factors are likely to influence spontaneous synaptic activity. Many afferent axons are cut during the preparation of the slice; all the afferents fibers from other parts of the brain and brainstem and from hypothalamic regions (Swanson and Sawchenko, 1983) not contained in the slice are obviously cut. Axons from some local cells that project to the supraoptic nucleus through a trajectory outside the plane of the slice are also cut. Part of the strong GABAergic input (van den Pol, 1985) to the supraoptic nucleus has been proposed to originate from GABA neurons located around the nucleus itself (Theodosis et al., 1986); therefore, GABAergic neurons projecting to supraoptic cells may still be present in the slice.

The striking differences that we observed between cells in terms of the frequency of events and the proportion of EPSCs versus IPSCs may be the result of different cells having different local input. The largest EPSCs and IPSCs were most likely due to action potentials originating from cut axons or from local circuits containing the soma of the projecting cell and its axon. The bursts of IPSCs that we observed in some cells are likely to be caused by bursts of action potentials in a GABAergic cell projecting to the recorded cell. Therefore, GABAergic inhibitory and glutamatergic excitatory local circuits probably exist and are functional in the slice.

Conclusions

The concept of neurosecretion was established four decades ago (Scharrer and Scharrer, 1954), and the supraoptic magnocellular neuroendocrine cells are the classic model of neurosecretory cells. Possible difference between conventional neurons and neuroendocrine cells in terms of passive membrane properties and synaptic transmission has been a long-standing question (Finlayson and Osborne, 1975; Mason and Bern, 1977; Yagi and Iwasaki, 1977). In the present study, the identification of neuroendocrine cells was unequivocal because we used direct visual identification of the supraoptic nucleus, and staining of cells showed that we were recording from cells in the nucleus. As reviewed by Renaud (1987, 1991), the intrinsic and synaptic properties that have been described in neuroendocrine cells are similar or identical to what is known to occur in neurons. Our observations on spontaneous EPSCs and IPSCs also support the hypothesis that neuroendocrine cells integrate electrical and chemical signals similarly to regular neurons. In particular, the present results support the hypothesis that, in the *in vitro* slice preparation, (1) glutamate mediates all the fast spontaneous EPSCs to magnocellular neuroendocrine cells, and (2) GABA acting on $GABA_A$ receptors mediates all the fast spontaneous IPSCs. The other neuroactive amines and peptides proposed as neurotransmitter candidates in the magnocellular neuroendocrine system are perhaps not neurotransmitters such as defined in the neuromuscular junction (Katz, 1969), but rather neuro-

modulators. By changing cell membrane potential and input resistance over long periods of time, they influence directly the amplitude and duration of the fast synaptic currents and therefore indirectly modulate the secretion of oxytocin and vasopressin.

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Homogeneity of Intracellular Electrophysiological Properties in Different Neuronal Subtypes in Medial Preoptic Slices Containing the Sexually Dimorphic Nucleus of the Rat

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ABSTRACT

The sexually dimorphic nucleus of the preoptic area (SDN-POA) is larger in male than in female rats, the male phenotype requiring the presence of circulating androgens perinatally. These experiments investigated the intracellular electrophysiology and morphology of SDN-POA neurons and compared these properties with those of other medial preoptic area (MPOA) neurons. Biocytin-injected cells in the SDN-POA either had one or two primary dendrites, or they had multipolar dendritic arrays; dendrites were aspiny or sparsely spiny and displayed limited branching. Neurons in other parts of the MPOA were similar morphologically. Regardless of morphology, neurons situated in either the SDN-POA or surrounding MPOA had low-threshold potentials and linear or nearly linear current-voltage relations. In most (73%) cells, stimulation of the dorsal preoptic region evoked a fast excitatory postsynaptic potential followed by a fast inhibitory postsynaptic potential (IPSP). Bicuculline blocked the fast IPSPs, which reversed near the Cl^2 equilibrium potential (-71 ± 5 mV), indicating their mediation by gamma-aminobutyric acid $(GABA)_A$ receptors.

Neurons in the SDN-POA have electrophysiological properties similar to those of other medial preoptic cells. When compared with the hypothalamic paraventricular nucleus, the MPOA appears relatively homogeneous electrophysiologically. This is despite the morphological variability within this population of neurons and heterogeneities that are also apparent at other levels of analysis. Finally, GABA-mediated, inhibitory synaptic contacts are widespread among medial preoptic neurons, consistent with indications from earlier reports that GABA provides a link in the feedback actions of gonadal steroids on the release of gonadotropic hormones.

Key words: intracellular recording, biocytin, low-threshold Ca^{2+} spikes, gamma-aminobutyric acid

The medial preoptic area (MPOA) is a hypothalamic region that plays a critical role in regulating diverse physiological processes, including fluid volume (van Gemert et al., 1975; Swanson and Mogenson, 1981), core temperature (Boulant, 1980), and reproduction (Giantonio et al., 1970; see Gorski, 1985). The firing rates of medial preoptic neurons are sensitive to osmotic stimuli, glucose, temperature changes, and gonadal steroids (Boulant and Silva, 1989). Lesions of the MPOA prevent phasic patterns of gonadotropic hormonal activity that promote ovulation in rodents (Gorski, 1985). Lesioning the MPOA also reduces male copulatory and ejaculatory behaviors (Giantonio et al., 1970), and it disrupts such maternal behaviors as nest building, pup retrieval, and nursing (Jacobson et al., 1980; Cohn and Gerall, 1989) in rats. The adult expression of these reproductive activities depends on the presence of gonadal steroids during development (Gorski, 1985). Steroid hormones also alter synaptic morphology (Raisman and Field, 1973) and cytoarchitectural subdivisions within the MPOA. The male version of the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is larger than that of the female (Gorski et al., 1980), requires circulating androgens perinatally (Jacobson et al., 1981).

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Anatomical and neurochemical techniques have been used extensively to study the MPOA from neuroendocrine and developmental perspectives. The animal model for most of this work has been the rat. In this species the MPOA is anatomically complex and has several subdivisions, including three subdivisions of the medial preoptic nucleus, the largest and most pronounced cell-dense region of the MPOA. The SDN-POA contains one of these subdivisions, the medial preoptic nucleus centralis (Simerly and Swanson, 1986), but also extends farther rostrally (Bloch and Gorski, 1988). In addition, the MPOA contains neurons that synthesize a variety of neurotransmitters and peptides (Simerly et al., 1986). Whether neurons in the SDN-POA have unique electrical properties when compared with other medial preoptic neurons is presently unknown. It is also not known whether the marked anatomical and neurochemical heterogeneity within the MPOA is associated with a similar degree of heterogeneity in electrophysiological properties.

Certain intrinsic electrical properties and anatomical characteristics have been useful in defining cell types in other hypothalamic nuclei. For example, three categories of ventromedial neurons, each possibly subserving a separate biological function, were identified on the combined basis of their intracellular electrical properties and neuronal morphology (Minami et al., 1986a,b). Also, in the region of the paraventricular nucleus of the rat, magnocellular neurons, parvicellular neurons, and cells surrounding the nucleus were found to have distinct and identifying membrane properties. These included the capacity for low-threshold Ca^{2+} spikes (LTS), the linearity versus nonlinearity of current-voltage (I-V) relations, and membrane time-constant values (Hoffman et al., 1991; Tasker and Dudek, 1991). Alternatively, despite anatomical heterogeneity, the MPOA could be comprised of neurons with mostly similar intrinsic membrane properties. In either situation, knowledge of these electrophysiological properties is essential for a rigorous understanding of the neural factors that regulate reproduction and other biologically important processes associated with this region.

Synaptic transmission in the SDN-POA also has not been studied and has been little examined in the MPOA as a whole. Gamma-aminobutyric acid (GABA) is likely to be the dominant inhibitory neurotransmitter in the hypothalamus (van den Pol et al., 1990). Spontaneous and evoked IPSPs that are mediated by GABA have been identified in other hypothalamic regions, including the paraventricular (Tasker and Dudek, 1993), supraoptic (Randle et al., 1986), and suprachiasmatic (Kim and Dudek, 1992) nuclei. GABA may also be important for functions attributed to the MPOA and may mediate inhibitory synaptic transmission in this area. Regional GABA concentration is relatively high in the MPOA (Mansky et al., 1982), and many medial preoptic neurons stain for the GABA-synthesizing enzyme glutamate decarboxylase (Flügge et al., 1986). GABA neurons in the MPOA may provide an important link in the feedback actions of gonadal steroids on the release of gonadotropic hormones from the anterior pituitary (Jarry et al., 1991). Putative inhibitory postsynaptic potentials (IPSPs) have been identified in the MPOA of mice (Hodgkiss and Kelly, 1990) and rats (Curras et al., 1991), though neither study pharmacologically evaluated whether these events were due to GABA release. Collectively, these findings suggest that GABA-mediated inhibitory synaptic contacts are widespread throughout the MPOA. However, this remains to be determined pharmacologically during recordings from this region.

The present study compared the intrinsic and synaptic electrical properties of neurons in the SDN-POA, as defined by Bloch and Gorski (1988), with those of surrounding medial preoptic cells. Hodgkiss and Kelly (1990) recorded intracellularly from medial preoptic neurons in mice and identified LTS potentials in approximately half of the cells in their sample. However, the focus of their study was to compare grafted preoptic cells with normal (control) neurons in tissue slices, and the MPOA of the mouse does not contain a cell group homologous to the SDN-POA (Bleier et al., 1982). Curras et al. (1991) recorded intracellularly from thermosensitive neurons in the MPOA and anterior hypothalamus of the rat, but they neither determined the precise cytoarchitectural location of their recordings nor investigated the presence or absence of LTS potentials. Therefore, by combining intracellular recording with intracellular staining and histology, we tested three hypotheses: 1) neurons in the SDN-POA are electrophysiologically homogeneous but differ from cells elsewhere in the MPOA; 2) some medial preoptic neurons generate LTS potentials; 3) GABA-mediated synaptic inhibition is widespread among these cells. Our results indicate that SDN-POA neurons do share common electrical properties, such as LTS potentials and GABA-mediated IPSPs; however, these properties are also common to other medial preoptic neurons. Some of these findings have been presented in abstract form (Hoffman et al., 1990).

MATERIALS AND METHODS

Slice preparation

Hypothalamic slices containing the MPOA were obtained from adult Sprague-Dawley rats (150–300 g, Charles River Breeding Laboratory) during the light phase of a 12-hour light/dark cycle. Animals were anesthetized with Nembutal (100 mg/kg intraperitoneal) and decapitated; their brains were quickly removed and placed in chilled (1–4°C), oxygenated, artificial cerebrospinal fluid (ACSF). This consisted (in mM) of 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.3 $MgSO_4$, 1.4 NaH_2PO_4, 2.4 $CaCl_2$, and 11 glucose. A tissue block containing the hypothalamus was dissected and sectioned coronally at 400 μm on a vibroslice (Campden Instruments). The anterior commissure, optic chiasm, and third ventricle were landmarks used to identify the MPOA. Slices containing the MPOA (Fig. 1) were placed in a ramp-type recording chamber, in which an interface between a humidified mixture of 95% O_2 and 5% CO_2 and the perfused ACSF (pH 7.4; 34 ± 1°C) was maintained. Slices were allowed to equilibrate in the recording chamber for 2–3 hours before recording.

Electrophysiological techniques

Micropipettes for intracellular recording were pulled from glass capillaries (1.0 mm OD, 0.5 mm ID, American Glass Co.), by using a Flaming-Brown puller; they were filled with either 2 M K-acetate or 2 M K-acetate containing 2% biocytin (Sigma) and had tip resistances of 90–200 MΩ. Microelectrodes were advanced in 4 μm steps with a piezoelectric microdrive (Nanostepper), and cell impalements were achieved by oscillating the negative-capacitance feedback. Electrical signals were recorded with an electrometer (model IR183, Neurodata Instruments or Axoclamp-2A, Axon Instruments), which contained a bridge circuit.

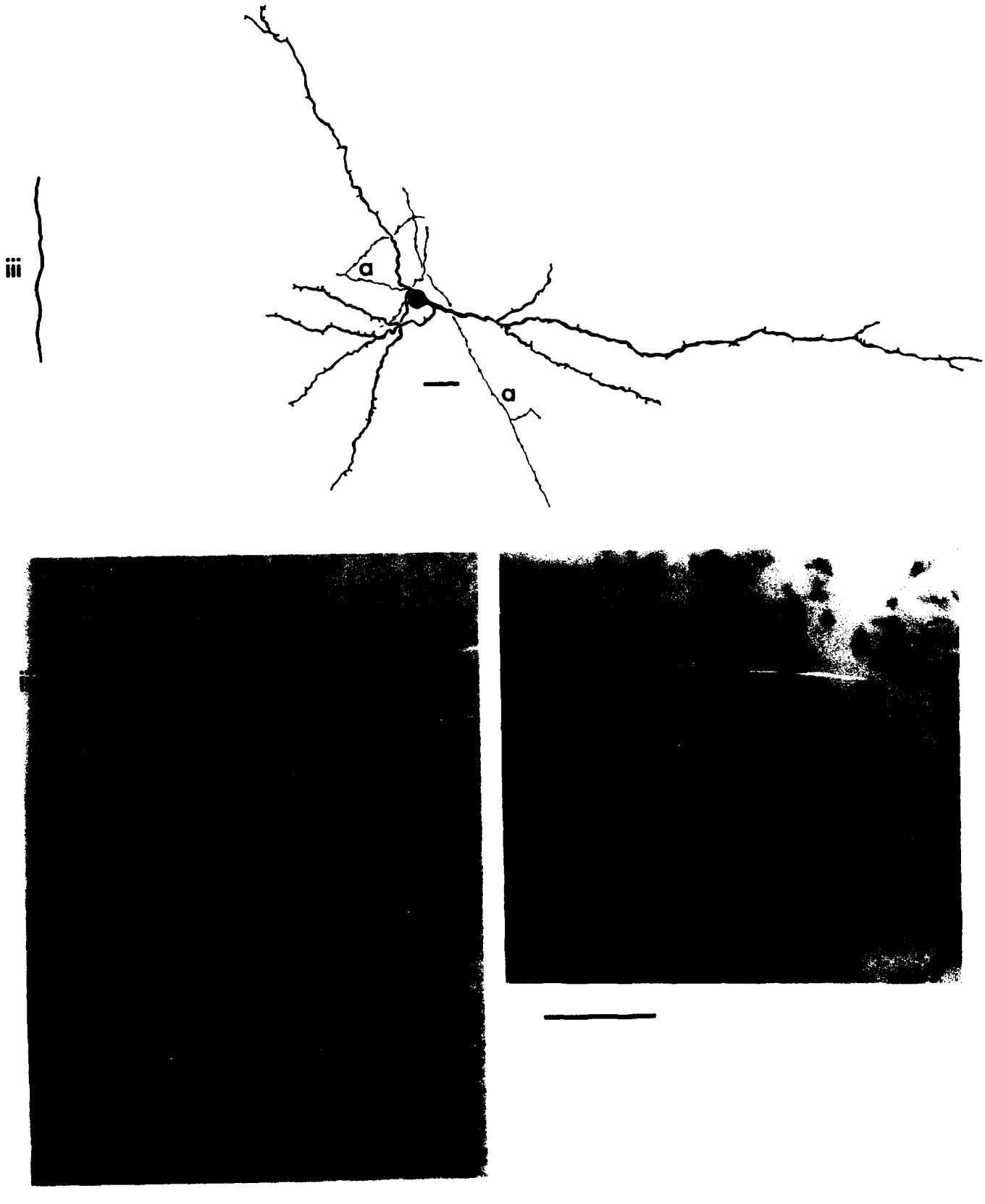

Figure 1

Signals were stored on video cassettes, by use of an analogue to digital converter (Neurocorder model DR-484, Neurodata Instruments), and traces were generated (ISC67AVE system, RC Electronics) and printed on an X-Y plotter or a laser printer.

Stimulating electrodes were made from insulated platinum-iridium wire (diameter, 75 μm). Constant-current stimulation was applied extracellularly to the dorsal preoptic region in a series of intensities (5–700 μA, 0.5 ms, 0.3 Hz) to evoke a complete range of synaptic responses. This stimulation site was chosen because it contains afferents to the medial preoptic nucleus (Simerly and Swanson, 1986) and because stimulation applied to this site consistently elicited synaptic responses in recorded neurons.

Drug application

In some experiments bicuculline methiodide (10 and 50 μM) was added to the ACSF perfusate to test whether GABA receptors mediated the IPSPs. Fast Na^+ spikes were blocked with bath application of tetrodotoxin (TTX, 50 μg/ml). Calcium-dependent potentials were challenged by replacing the ACSF with a solution containing (in mM) 125.5 NaCl, 3 KCl, 1.3 $MgCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 11 glucose, 0.2 $CaCl_2$, and 0.5 $NiCl_2$.

Anatomy

Biocytin was iontophoresed intracellularly with negative current pulses (2 Hz, 100–300 ms, 200–500 pA), and injected cells were histologically processed as previously described (Horikawa and Armstrong, 1988; Hoffman et al., 1991; Tasker et al., 1991). Briefly, slices were immersion-fixed for at least 12 hours in 4% paraformaldehyde in 0.1 M Na-cacodylate buffer (pH 7.25; 5°C) and sectioned on a sliding microtome. An avidin-biotinylated horseradish peroxidase bridge was attached (Vectastain Elite kit, Vector Laboratories) and an opaque reaction product formed using a glucose-oxidase procedure and diaminobenzidine (−4 HCl) (Sigma) as the chromogen (Smithson et al., 1984). Sections were mounted onto gelatin-coated slides and counterstained with cresyl violet or toluene blue.

Statistical analyses

Whether neurons of the SDN-POA differ from other medial preoptic neurons in the electrophysiological parameters examined in the present study was determined using Student's t-test. Power analyses assessed the adequacy of our sample sizes in these comparisons for revealing differences in parameters that define cell types in the ventromedial hypothalamus (Minami et al., 1986a) and the region of the hypothalamic paraventricular nucleus (Tasker and Dudek, 1991). The power analyses were based on magnitudes of effects and standard deviations reported by Minami et al. (1986a) and Tasker and Dudek (1991). The presence versus absence of low-threshold potentials helps define ventromedial hypothalamic and paraventricular cell types. The reported percentages of neurons with low-threshold potentials in these regions (Minami et al., 1986a; Tasker and Dudek, 1991) were the expected values to which we compared (χ^2) our obtained percentage of medial preoptic neurons that had these potentials. This comparison further assessed electrophysiological homogeneity in the MPOA relative to other hypothalamic regions.

Medial preoptic neurons were assigned to two categories on the basis of their dendritic arbor (see below). Whether the SDN-POA was uniquely associated with a dendritic morphology or soma size, compared with other medial preoptic neurons, was determined using Fisher's exact test and Student's t-test, respectively. Finally, whether the two categories of morphology differed electrophysiologically was assessed using t-tests. A type-I error probability ≤ 0.05 was the criterion in all statistical analyses performed in this investigation.

RESULTS

Intracellular recordings were obtained from 46 medial preoptic neurons, all of which had overshooting action potentials ≥ 50 mV measured from threshold to peak (mean ± SEM = 61.2 ± 1.6 mV) and apparent resting potentials at or more negative than −50 mV (mean = −60.7 ± 2.2 mV, where each resting potential could be verified at the end of an experiment, n = 33). All recordings included in this study were stable for at least 10 minutes. Most of the recordings (94%) were made in slices from males; however, three medial preoptic neurons from ovariectomized females were included in analyses of LTS potentials, which were similar to those recorded in male tissue.

Medial preoptic subdivisions and neuronal morphology

After impalement with biocytin-filled electrodes and subsequent histological procedures, 24 neurons were recovered and located in the SDN-POA, the medial preoptic nucleus medialis (MPNm), and elsewhere in the MPOA. Stable recordings were obtained in 18 of these cells (Table 1). Neurons varied with respect to their dendritic arbor: one set of cells (46% of stained neurons) had one or two primary dendrites (Fig. 1), and another set (54%) had multipolar dendritic arrays (Fig. 2). These characteristics of morphology were not uniquely associated with specific medial preoptic subdivisions examined in the present study (Fig. 3). Soma sizes were similar in both sets of neurons: longest-axis soma diameters of neurons with one or two primary dendrites ranged from 11 to 34 μm and shortest-axis diameters from 10 to 22 μm. Soma diameters of multipolar cells ranged from 16 to 34 μm (longest axis) and from 10 to 16 μm (shortest axis). Dendritic arbors in both sets of neurons were sparsely spiny or aspiny; only primary dendrites were observed on 38% of stained cells and both primary and secondary dendrites on 62% of the cells. Axons typically originated from primary dendrites and most often coursed in a medial to ventromedial direction (seven of ten observations), in some cases sending off local collaterals (Fig. 4). Both primary axons and local collaterals contained varicosities (Figs. 1, 2, and 4). Regardless of cytoarchitectonic location or morphology, stained neurons had similar electrical properties ($P > 0.05$) that did not differ from those recorded in most unstained (or nonanatomically recovered) neurons (see Table 1 and below).

Fig. 1. Morphology of a bipolar medial preoptic neuron. Camera lucida drawing (**top**) and photomicrographs (**bottom**) showing a biocytin-injected neuron with two primary dendrites. As shown at lower power (left), this cell (arrowhead) was situated in the sexually dimorphic nucleus of the preoptic area. iii, third ventricle. Calibration bars = 50 μm.

TABLE 1. Basic Electrophysiological Properties of Medial Preoptic Neurons[1]

	SDN-POA	MPOA	Undetermined location
Resting potential (mV)	−57.8 ± 1.7 (6)	−61.9 ± 2.8 (10)	−61.2 ± 2.1 (17)
Input resistance (MΩ)	164 ± 15 (5)	191 ± 24 (9)	209 ± 19 (16)
Membrane time constant (ms)	16.8 ± 4.5 (5)	13.8 ± 2.1 (8)	15.6 ± 1.7 (10)
Spike amplitude from threshold (mV)	61.1 ± 2.6 (7)	62.3 ± 1.7 (11)	60.7 ± 1.3 (27)
Spike duration at half amplitude (ms)	0.71 ± 0.09 (7)	0.83 ± 0.08 (10)	0.78 ± 0.05 (21)

[1]Properties are expressed as mean ± SEM (n). No statistically significant differences were observed (Student's t-test, $P > 0.05$) between neurons in the sexually dimorphic nucleus of the preoptic area (SDN-POA) and those situated in other parts of the medial preoptic area (MPOA). Recorded neurons in the undetermined location category were either not injected with biocytin or were not histologically recovered after injection. The MPOA is the probable site of these recordings (note the similarity between these values and those of stained neurons in the SDN-POA and elsewhere in the MPOA).

Low-threshold potentials

The most salient intrinsic property of medial preoptic neurons was their capacity to generate low-threshold potentials (mean amplitude ± SEM = 29.2 ± 1.8 mV), as observed in 98% of all recorded cells (n = 46) and 100% of those neurons that were stained and histologically identified within the SDN-POA and elsewhere in the MPOA. This percentage (98%) is significantly higher than expected ($P < 0.001$) based on the reported percentages of cells with low-threshold potentials in the ventromedial hypothalamus (50%) (Minami et al., 1986a) and region of the paraventricular nucleus (68%) (Tasker and Dudek, 1991).

Low-threshold potentials were observed in the present study by first hyperpolarizing medial preoptic neurons, and then applying depolarizing current pulses. This activated low-threshold potentials that typically generated up to three Na^+ spikes (Fig. 5A,B). The low-threshold potentials persisted during TTX block of fast, voltage-dependent Na^+ channels (n = 1) but were blocked by bath application of Ni^{2+} in low-$[Ca^{2+}]_o$ HEPES medium (n = 3) (data not shown). These potentials were also observed as anodal-break spikes following the offset of hyperpolarizing current pulses (Fig. 5C, D). Anodal-break spikes were also blocked by Ni^{2+} in the low-$[Ca^{2+}]_o$ medium. Varying the duration of the hyperpolarizing current pulse revealed that voltage-dependent deinactivation of the low-threshold conductance was also time dependent (Fig. 5C). Thus we refer to these low-threshold events as putative low-threshold Ca^{2+} spikes (LTS).

In some medial preoptic neurons, LTS potentials appeared to underlie membrane oscillations, which followed the offset of hyperpolarizing current pulses and resultant anodal-break spikes (Fig 5D). These oscillations were not observed in all cells.

Other membrane responses to current injection

Along with having LTS potentials, medial preoptic neurons were similar in terms of other intrinsic properties (Table 1). Power analyses combined with t-tests indicated that the magnitude of differences in membrane time constant between cell types in the ventromedial hypothalamus (Minami et al., 1986a) and region of the hypothalamic paraventricular nucleus (Tasker and Dudek, 1991) did not exist between SDN-POA neurons and other medial preoptic cells (Table 1). Such a difference would have been detected with sample sizes of three (based on Minami et al., 1986) or six (based on Tasker and Dudek, 1991) neurons. However, mean time constant did not significantly differ between SDN-POA neurons and other medial preoptic cells ($P > 0.05$). A difference in action-potential amplitude between SDN-POA cells and other medial preoptic neurons similar to that between hypothalamic paraventricular cell types (Tasker and Dudek, 1991) would have been revealed with sample sizes of eight neurons. However, we did not observe such a difference ($P > 0.05$). The sample sizes used in the present study (Table 1) were similar to the values indicated in these power analyses.

Most medial preoptic neurons (n = 25) had I-V relations that were linear, with little or no evidence of anomalous and time-dependent inward rectification at hyperpolarizing potentials up to and greater than 40 mV below resting potential (mean Vm ± SEM = −61.1 ± 1.6 mV) [Fig. 6A,B (left)]. The hyperpolarizing I-V relations of only two neurons suggested inward rectification, and the I-V plots from these cells were only weakly curvilinear [Fig. 6B (right)]. One of these neurons was otherwise electrophysiologically similar to other recorded cells in this study (e.g., capacity for LTS potentials); however, the other cell was the only recorded neuron that failed to generate an obvious LTS potential (data not shown). This neuron was not injected with biocytin, and so its position within the MPOA could not be verified.

Depolarizing current pulses of different intensity were applied to evaluate repetitive firing of medial preoptic cells (n = 21). In most neurons (88%), spike frequency increased as a function of current intensity; spike amplitude and frequency declined as a function of time during the pulse (i.e., spike-frequency adaptation). The degree of adaptation was variable across neurons, and only one or two action potentials could be evoked in two cells. In most neurons (83% of 24 cells), relatively small afterhyperpolarizations (AHP) followed spike trains (following 7–11 spikes, mean ± SEM amplitude = −4.7 ± 0.5 mV; duration = 64 ± 13 ms, measured at half-maximum amplitude). Mean (±SEM) AHP = −3.9 ± 0.8 mV in the SDN-POA (n = 4) and −3.9 ± 1.2 in other stained medial preoptic neurons (n = 7). Detection of a difference between these two cell groups similar to that between ventromedial hypothalamic cell types (Minami et al., 1986a) required sample sizes of four neurons (power analysis). No such difference was observed ($P > 0.05$), even though the smaller sample in this comparison (SDN-POA) contained four neurons.

Synaptic responses

Extracellular stimulation (50–700 μA) of the dorsal preoptic region reliably evoked synaptic potentials (n = 34 neurons). In most cells (73%), this stimulation produced a fast excitatory postsynaptic potential (EPSP) that was attenuated by a single fast IPSP (Figs. 7A, 8). Average latencies (±SEM) for evoked EPSPs and IPSPs were 2.7 ± 0.2 ms and 4.8 ± 0.5 ms, respectively. Although a single IPSP was most typically observed (Fig. 8A), stimulation evoked multiple IPSPs in a few neurons (Fig. 7A, top trace,

Fig. 2. Morphology of a multipolar medial preoptic neuron. Camera lucida drawing (**left**) and photomicrographs (**center and right**) showing a biocytin-injected neuron with a multipolar array of primary dendrites. This neuron (arrowhead, lower power photomicrograph) was located in the medial preoptic nucleus medialis. iii, third ventricle. Calibration bars = 50 μm.

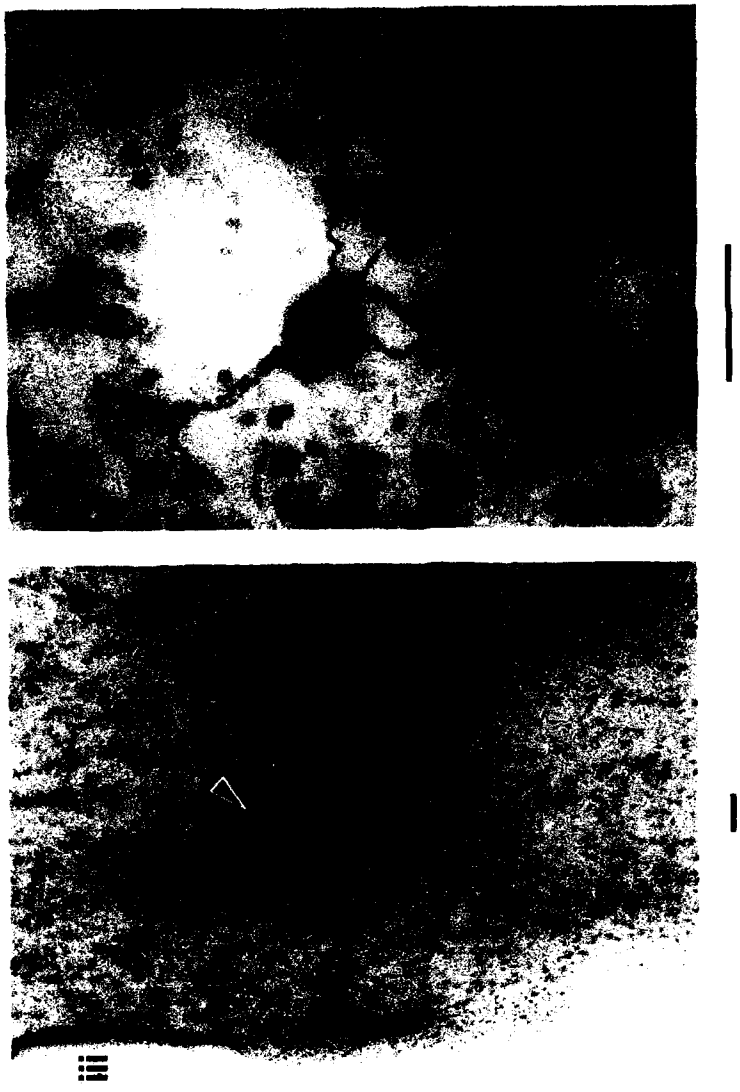

Figure 2

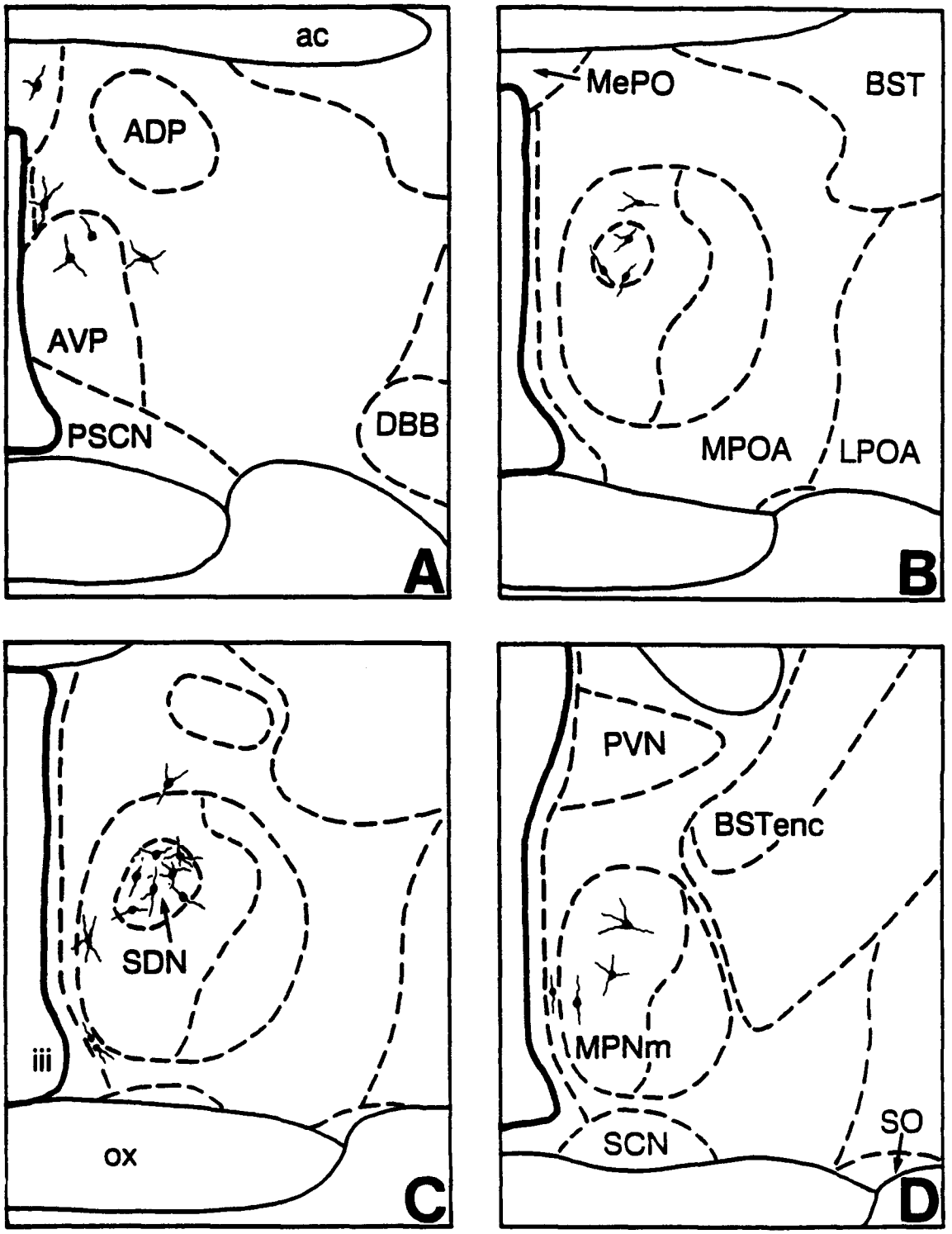

Fig. 3. Schematic diagram of the approximate location of recorded and stained neurons with respect to medial preoptic subdivisions. **A–D:** Rostral-caudal progression through the medial preoptic area (MPOA). Stained neurons are schematicized in terms of their numbers of primary dendrites. ac, anterior commissure; ADP, anterodorsal preoptic nucleus; AVP, anteroventral periventricular nucleus; BST, bed nucleus of the stria terminalis; BSTenc, BST encapsulated portion; DBB, diagonal band of Broca; LPOA, lateral preoptic nucleus; MPNm, medial preoptic nucleus medialis; MePO, median preoptic nucleus; ox, optic chiasm; PVN, paraventricular nucleus; PSCN, preoptic suprachiasmatic nucleus; SDN, sexually dimorphic nucleus of the preoptic area; SCN, suprachiasmatic nucleus; SO, supraoptic nucleus; iii, third ventricle. Schematics were adapted with permission from Simerly and Swanson, 1986.

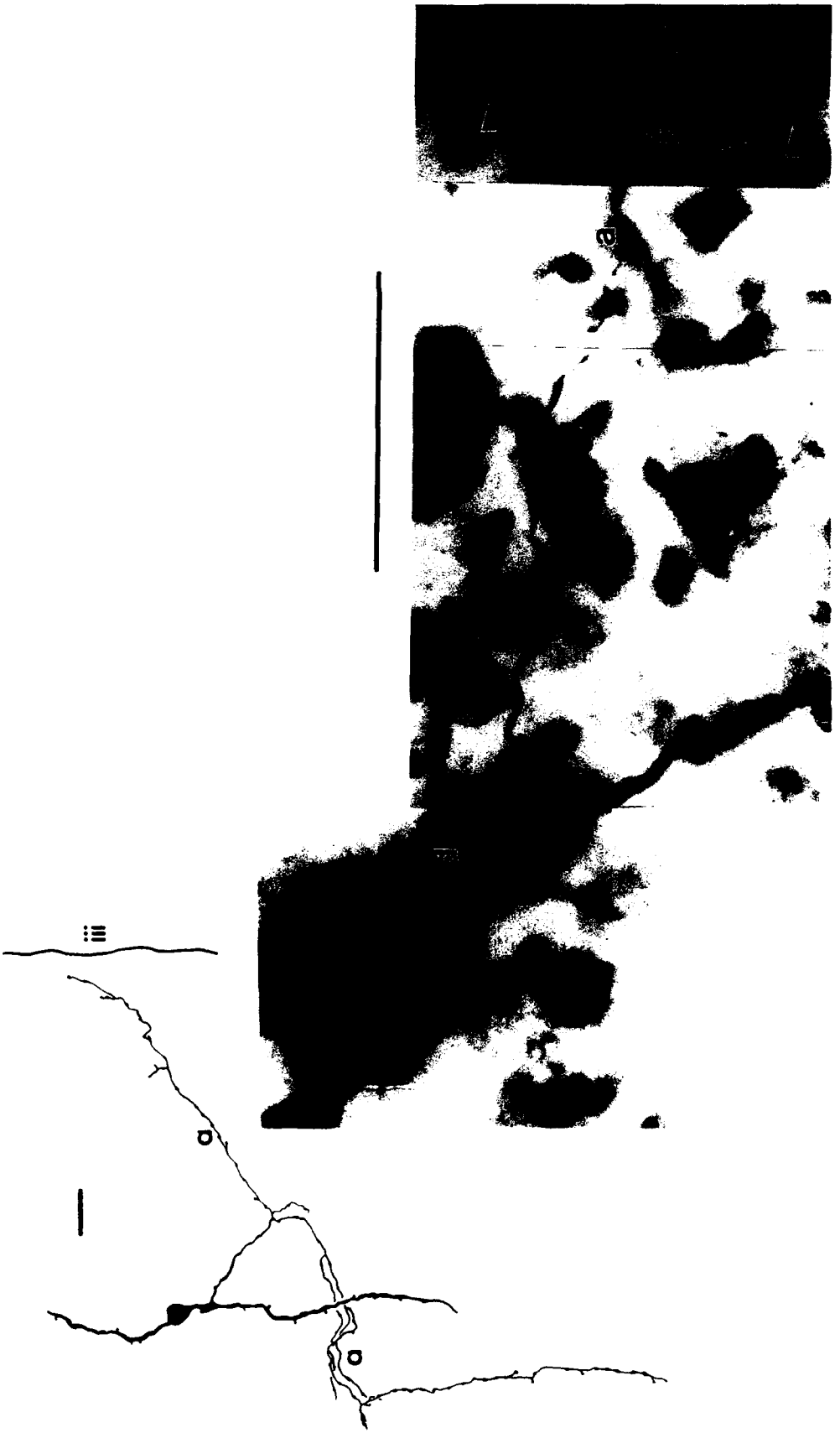

Fig. 4. Axon collaterals from a medial preoptic neuron. Camera lucida drawing and photomicrograph of a bipolar neuron located in the sexually dimorphic nucleus of the preoptic area. Note that its axon (a) arose from a primary dendrite (d) and ramified locally. Axon collaterals were covered with varicosities (arrowheads). iii, third ventricle. Calibration bars = 50 μm.

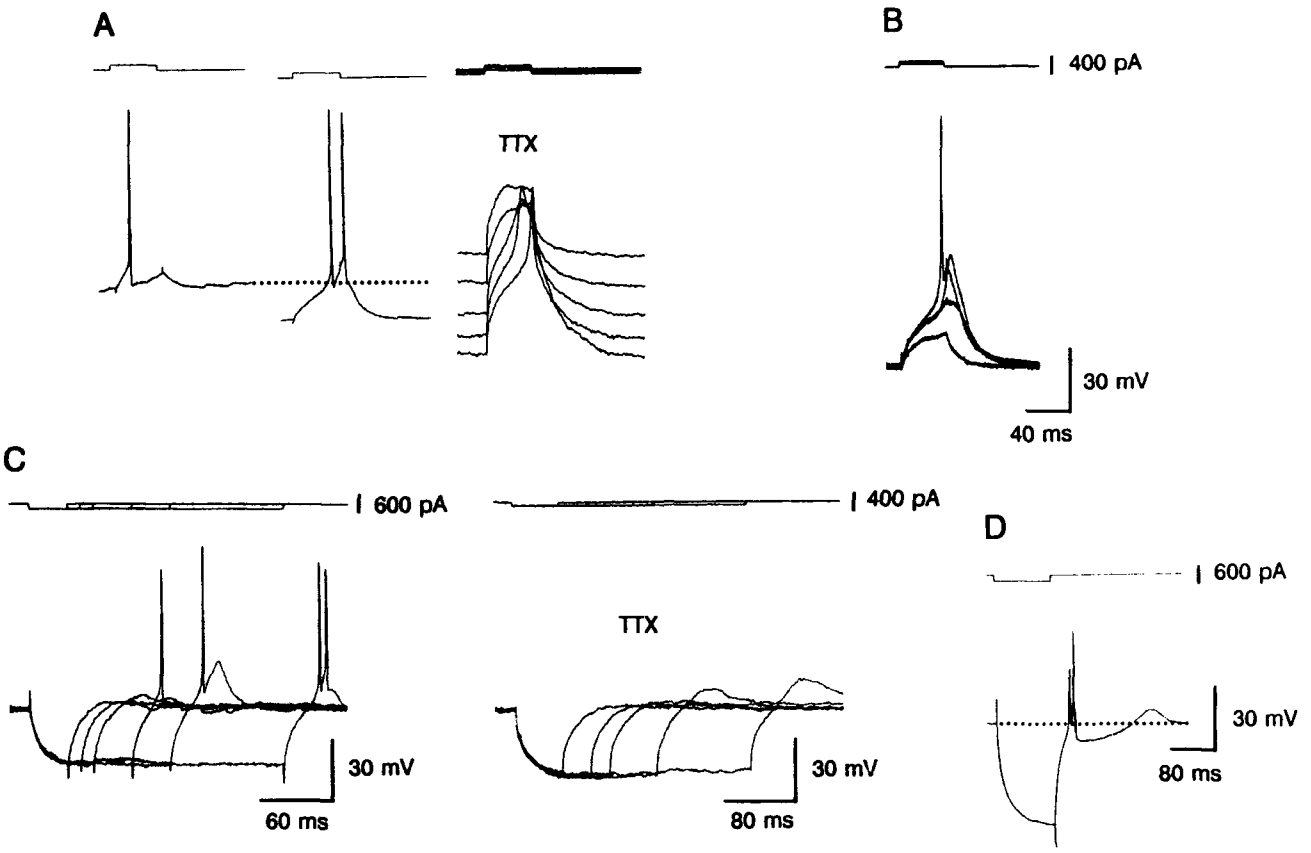

Fig. 5. LTS potentials recorded in medial preoptic neurons. **A:** At rest a neuron fired a single action potential in response to the injection of a depolarizing current pulse (left). As a neuron was hyperpolarized with steady (DC) current, the same current pulse intensity triggered a regenerative depolarization to Na^+-spike threshold, resulting in a short burst of action potentials (middle). The size of this depolarization grew with increasing DC hyperpolarization, shown here during bath application of tetrodotoxin (TTX; 0.5 μg/ml) to a different neuron (right). **B:** Progressively increasing the intensity of a depolarizing current pulse from a hyperpolarized condition also progressively increased the size of the LTS potential (calibration bars in B also apply to A). **C:** Time dependence of LTS potentials. Hyperpolarizing current pulses had to be sufficiently long for an LTS potential to occur with the offset of the pulse, whether the Na^+ spikes were unblocked (left) or blocked (right) with TTX. **D:** An anodal-break spike could generate a burst of Na^+ spikes, and a post-burst after hyperpolarization could then trigger a membrane oscillation (or series of oscillations) (voltage trace in D is the average of four traces). In this and in subsequent figures, top traces are current, the bottom traces are voltage, and dotted lines indicate resting potential. Traces in A (right) and C (right) are from a female rat.

and B). In some instances spikes occurred at the termination of large IPSPs (Fig. 7B), suggesting that the IPSPs deinactivated the conductance underlying the LTS potentials. Spontaneous PSPs were frequently recorded, and among these events IPSPs were prominent (Fig. 7C). The reversal potential of evoked IPSPs was -71 ± 5 mV (mean ± SEM, n = 7) (Fig. 8A), and bath application of bicuculline (10 and 50 μM) blocked the evoked IPSPs (n = 3). In neurons in which stimulation usually evoked only IPSPs in normal medium, bicuculline blocked the IPSPs and revealed EPSPs (Fig. 8B).

DISCUSSION

General

To our knowledge, the present investigation is the first to study the intrinsic membrane properties of neurons histologically verified to be situated in the SDN-POA. The hypothesis that sets of these properties were similar among SDN-POA neurons was confirmed, but the hypothesis that these properties differed between neurons in this nucleus with cells located elsewhere in the MPOA was not. Instead, neurons with a common set of intrinsic properties appear to constitute the predominant electrophysiological cell type across medial preoptic subdivisions. Unstained neurons, whose precise cytoarchitectonic locations could not be determined, also had properties similar to those of stained cells with verified locations within the MPOA. Thus data from the unstained neurons also support the concept that the MPOA is relatively homogeneous with regard to these properties.

This homogeneity is not due to the selection of cells residing in a single medial preoptic location or having a common morphology, because the intracellular staining confirmed that the data were obtained from a variety of locations and from cells with different morphology. Minami et al. (1986a) found that ventromedial hypothalamic neurons could be classified by their dendritic arrays, which correlated with distinct sets of electrophysiological properties, including the presence or absence of LTS potentials. In the present study, a similar range of dendritic patterns was observed among stained medial preoptic cells. However, neither this nor other morphological characteristics (e.g., soma size) correlated with defining sets of electrophysiologi-

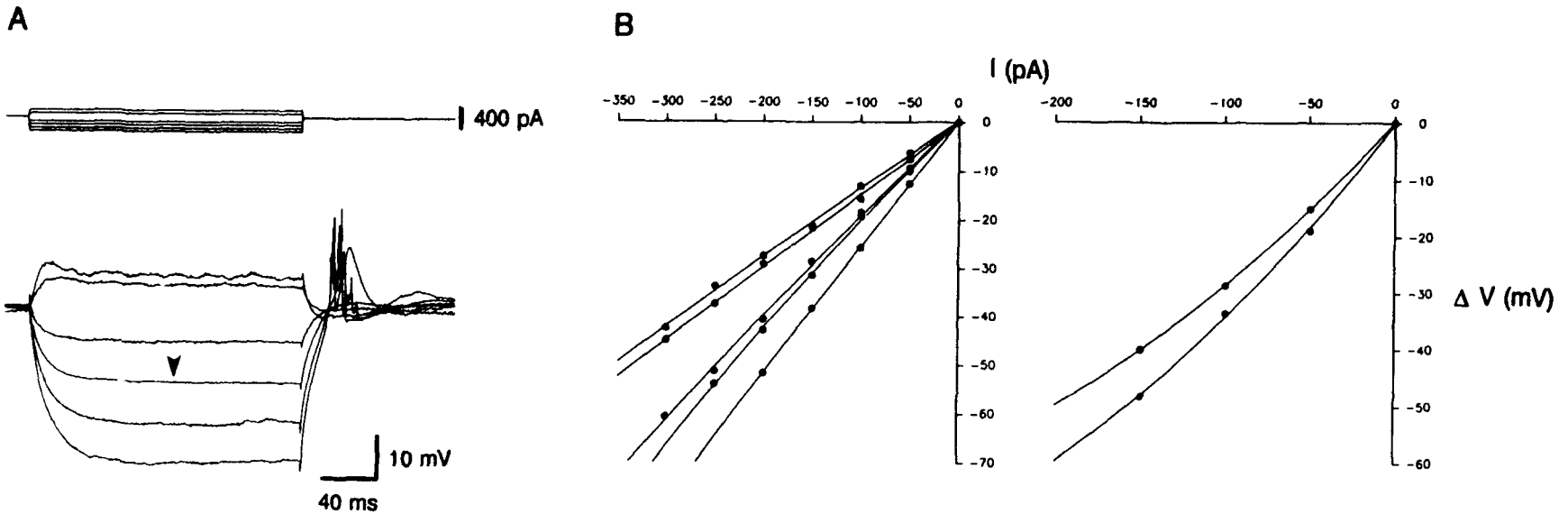

Fig. 6. I-V relations of medial preoptic neurons. Rectangular current pulses of 100–300 ms duration of increasing intensity were injected and the voltage responses recorded. Current pulses were typically injected from rest (mean ± SEM = 61.1 ± 1.6 mV) or occasionally from a slightly hyperpolarized (DC) condition to reduce spontaneous firing (64.7 ± 0.33 mV). Membrane time constant and input resistance values were calculated from averaged voltage deflections (n = 5–30) in response to weak current pulses (−50 pA or −100 pA) (see Table 1). **A:** Voltage deflections of a medial preoptic cell in response to current injections. **B (left):** I-V plots of several representative medial preoptic neurons. Both A and B (left) show linear I-V relations in the hyperpolarizing direction. **B (right):** I-V plots from the only two neurons with nonlinear I-V relations, suggesting inward rectification in the hyperpolarizing direction. Traces in A are the average of 4–7 traces except that indicated by the arrowhead, which was averaged across 22 traces for membrane time constant measurement.

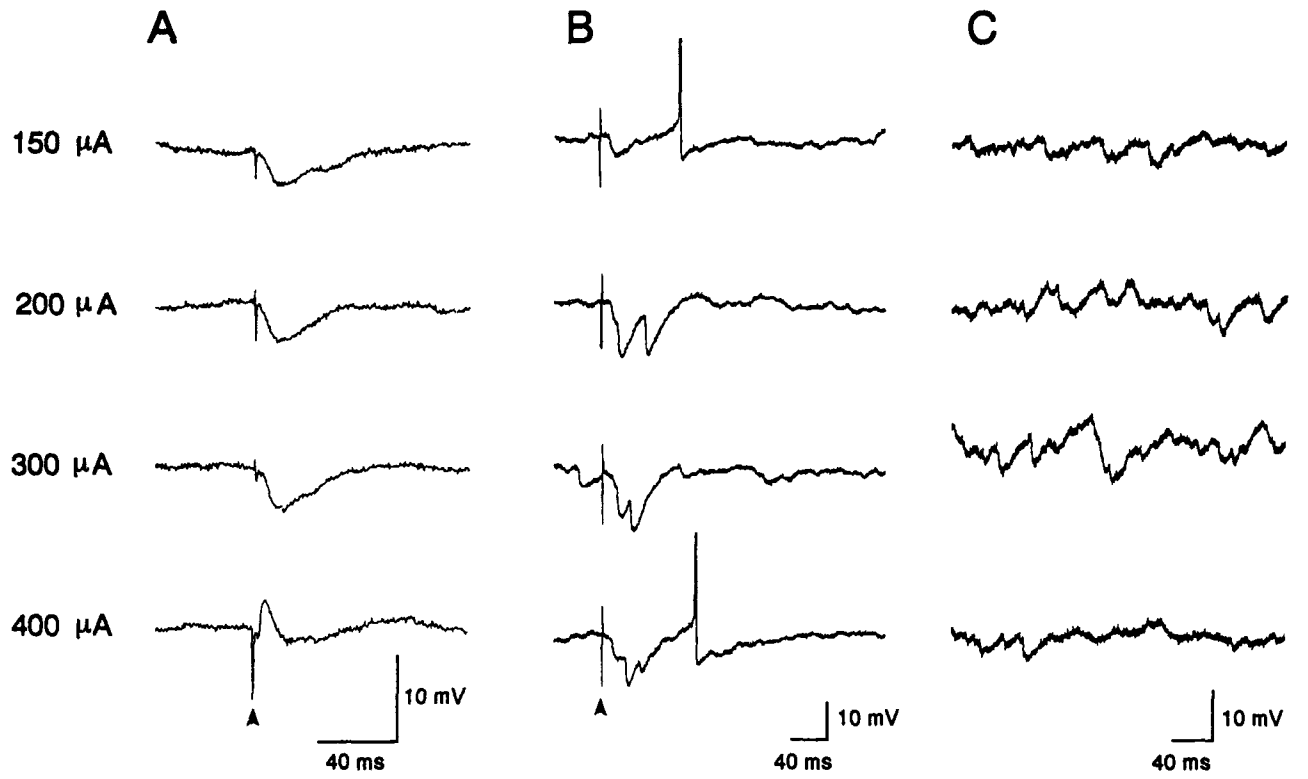

Fig. 7. Evoked and spontaneous postsynaptic potentials recorded in medial preoptic neurons. **A:** In most medial preoptic cells, an EPSP-IPSP sequence could be evoked by extracellular stimulation of the dorsal preoptic region but only at certain stimulus intensities. In this neuron, increasing stimulus intensity only slightly increased IPSP amplitude until an intensity was reached that evoked an EPSP-IPSP sequence (voltage traces are averaged across four traces). **B:** In a few medial preoptic cells, extracellular stimulation of the dorsal preoptic region evoked one or more IPSPs. The four traces are from the same neuron, in which multiple IPSPs were evoked by a 30 μA stimulus. Note that depolarizations generating action potentials (clipped in this figure) occasionally followed the large IPSPs. **C:** Spontaneous PSPs, including IPSPs, were frequently recorded in medial preoptic neurons, shown here by four separate traces from the same cell recorded at rest. Arrowhead in A and B indicates stimulus artifact.

cal properties. Thus neurochemical and anatomical heterogeneity within this region (Simerly et al., 1986; Simerly and Swanson, 1986) does not appear to be associated with a similar heterogeneity in neuronal membrane properties recorded under constant-temperature conditions.

This study does not rule out the possibility that a small subset of neurons with distinct electrophysiological properties also resides in the MPOA. However, our findings indicate that the degree of electrophysiological homogeneity of neurons in this region is similar to that of the supraoptic nucleus, although electrophysiological properties differ between medial preoptic and supraoptic cells (Andrew and Dudek, 1983; Andrew and Dudek, 1984a, b; Bourque and Renaud, 1985). In contrast, the hypothalamic paraventricular nucleus is more heterogeneous than either the MPOA or supraoptic nucleus. The three different cell types in or near the paraventricular nucleus were defined on the basis of the same electrophysiological properties examined in the present investigation (Hoffman et al., 1991; Tasker and Dudek, 1991).

LTS

As hypothesized, medial preoptic neurons could generate putative LTS potentials; however, the high percentage (98%) of cells with this capacity found in this region was surprising. To our knowledge, the MPOA is unique in this respect among hypothalamic regions studied to date. Unlike the paraventricular, ventromedial and arcuate nuclei (Minami et al., 1986a, b; Loose et al., 1990; Hoffman et al., 1991; Tasker and Dudek, 1991), where only subsets of neurons display LTS potentials, all anatomically identified medial preoptic neurons recorded in the present investigation had this capacity. This was also true for all but one unstained neuron. This finding provides further evidence for cell-type homogeneity in the MPOA of the rat; however, it appears to contrast with findings from grafted and nongrafted medial preoptic neurons in mice, in which LTS potentials were not identified in a large proportion of recorded cells (Hodgkiss and Kelly, 1990). This apparent discrepancy could reflect a species difference or, instead, the different focus of the previous study (Hodgkiss and Kelly, 1990).

The LTS potentials recorded in the present investigation generally were not as large as those described in the inferior olivary nucleus (Llinás and Yarom, 1981) and thalamus (Jahnsen and Llinás, 1984). Nonetheless, they had similar voltage dependence, and they were likewise Ca^{2+} dependent. These data, plus the voltage dependence of these potentials, suggest mediation by transient (T) Ca^{2+} currents, which are particularly sensitive to Ni^{2+} (Fox et al., 1987). In medial preoptic neurons, as in parvicellular (type II) neurons in the paraventricular nucleus (Hoffman et al., 1991; Tasker and Dudek, 1991), LTS potentials generated only one or a few Na^+ spikes. Also as recorded in paraven-

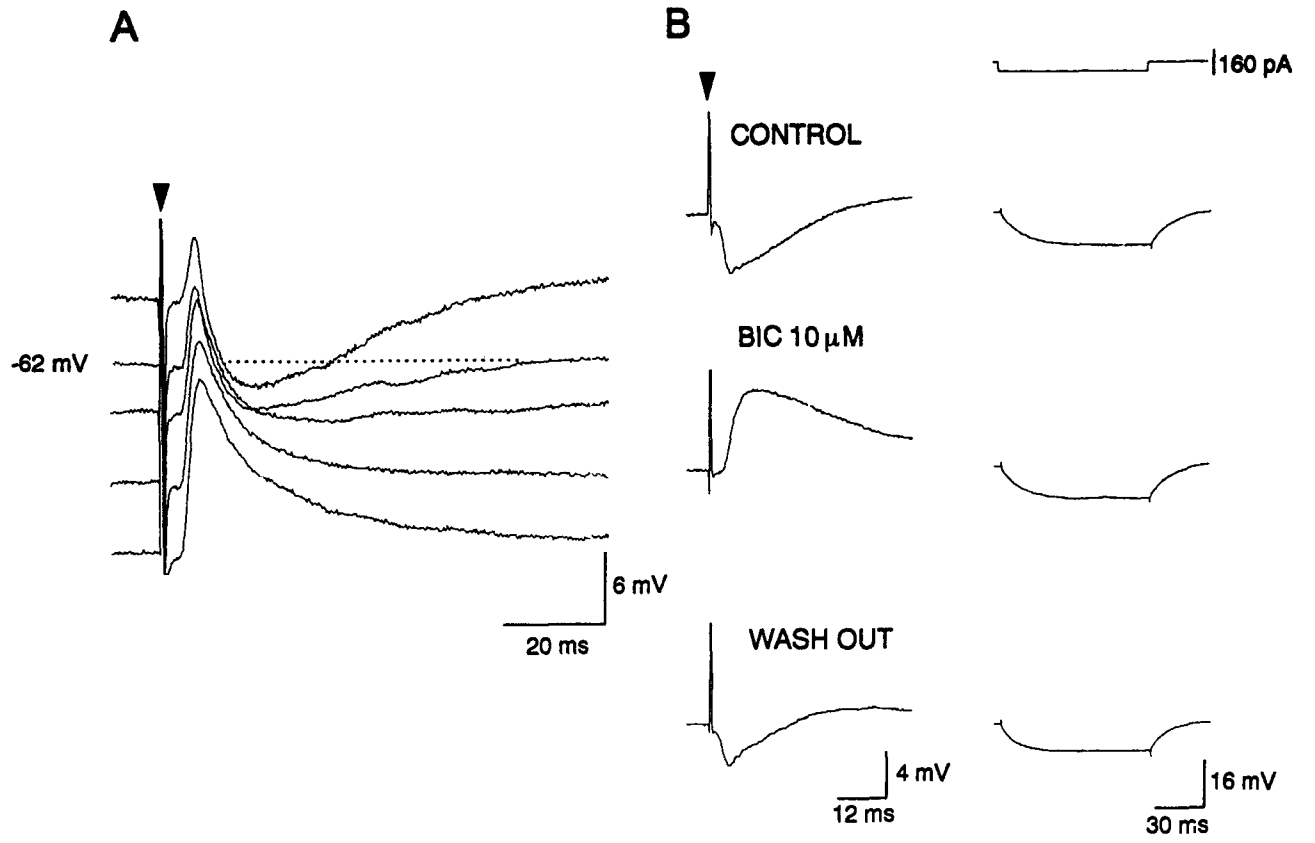

Fig. 8. Properties of evoked IPSPs. **A:** In this medial preoptic neuron, a 300 μA extracellular stimulus to the dorsal preoptic region evoked an EPSP-IPSP complex. The IPSP reversed near −72 mV. **B:** In another medial preoptic cell, a 200–300 μA stimulus to the dorsal preoptic region evoked only an IPSP (top) in normal medium. Bath application of the $GABA_A$ antagonist, bicuculline (BIC, 10 μM), blocked the evoked IPSP and revealed an evoked EPSP (middle). The effect of bicuculline was reversible (bottom) and was not accompanied by appreciable changes in input resistance (right traces). During this experiment, the cell was hyperpolarized with 11 pA of steady current to prevent spontaneous firing. Voltage traces in B are averaged across 21–30 individual traces. Arrowheads indicate stimulus artifacts.

tricular parvicellular neurons, LTS-mediated anodal-break spikes in medial preoptic neurons could trigger membrane oscillations. These oscillations, however, were not as robust as those reported for cells situated near but outside the paraventricular nucleus (type III neurons) (Hoffman et al., 1991; Tasker and Dudek, 1991). Other membrane properties described below also suggest similarity between medial preoptic and paraventricular parvicellular neurons.

Other intrinsic properties

Values for input resistance, membrane time constant, Na^+-spike amplitude and duration, and resting potential were similar among medial preoptic neurons. Most medial preoptic cells had linear I-V relations in the hyperpolarizing direction, with the I-V plots from two cells deviating slightly from linearity. Similar I-V relations were reported for paraventricular parvicellular neurons (Hoffman et al., 1991; Tasker and Dudek, 1991). This suggests three main points: 1) a broad category of hypothalamic parvicellular neurons with a common set of intrinsic electrophysiological properties extends across certain hypothalamic regions; 2) cells with these properties constitute the major neuronal type in the SDN-POA and surrounding MPOA; and 3) these properties are a substrate for integrating responses to the several homeostatic stimuli known to affect the activity of cells in this region (Boulant and Silva, 1989).

Fast synaptic events and GABA

Findings from this study support the hypothesis that medial preoptic neurons receive $GABA_A$-receptor-mediated synaptic inhibition. Spontaneous EPSPs and IPSPs were consistently recorded in these cells, and an EPSP-IPSP sequence could often be evoked by applying electrical stimuli to the dorsal preoptic region. The evoked IPSPs were usually superimposed on the EPSPs, thus reducing the amplitude and duration of the evoked EPSPs. In some experiments, evoked EPSPs could be observed only when the IPSPs were blocked with bicuculline. This antagonism of IPSPs by bicuculline, coupled with IPSP reversal near the Cl^- equilibrium potential (Randle et al., 1986), indicates that ligand-binding at $GABA_A$ receptors mediates these events. Previous findings suggest that medial preoptic cells synthesize GABA (Mansky et al., 1982; Flügge et al., 1986) and that GABA-releasing neurons participate in synchronizing phasic gonadotropic activity (Jarry et al., 1991). Our findings relate to this latter possibility in two ways: 1) GABA appears to be the primary, if not the only, neurotransmitter mediating fast IPSPs in medial preoptic neurons; and 2) the axons of stained cells often ramified into an apparent network of local collaterals, possibly forming local circuits in this region (see Fig. 4).

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COMMENTARY

CELLULAR COMMUNICATION IN THE CIRCADIAN CLOCK, THE SUPRACHIASMATIC NUCLEUS

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Abstract—The hypothalamic suprachiasmatic nucleus functions as the circadian clock in the mammalian brain. Communication between the cells of the suprachiasmatic nucleus is likely to be responsible for the generation and accuracy of this biological clock. Communication between many cells of the brain is mediated by action potentials that pass down the axon and cause release of neurotransmitters at the neuronal synaptic junction. Additional mechanisms of cellular communication appear to operate in the suprachiasmatic nucleus. Several lines of evidence point to multiple modes of cellular communication: these include the continuing operation of the clock after Na^+-mediated action potentials have been blocked, the orchestrated metabolic rhythms of suprachiasmatic nucleus cells prior to synaptogenesis, the entrainment of fetal to maternal rhythms, and the rapid recovery of function after suprachiasmatic nucleus transplants into arrhythmic rodents. Possible alternative means of intercellular communication in the suprachiasmatic nucleus are examined, including calcium spikes in presynaptic dendrites, ephaptic interaction, paracrine communication, glial mediation, and gap junctions. This paper identifies and examines some of the unanswered questions related to intercellular communication of suprachiasmatic nucleus cells.

CONTENTS

1. INTRODUCTION

Biological circadian rhythms are pervasive features of mammalian physiology and behavior. Converging lines of evidence indicate that the biological clock regulating mammalian circadian rhythms is in the suprachiasmatic nucleus (SCN), a small cluster of neurons above the optic chiasm (Fig. 1). Destruction of the SCN results in a loss of behavioral and physiological circadian rhythms,[100,155,171] as does isolation of the SCN from the rest of the brain by a knife cut that severs the efferent axons. The isolated SCN, on the other hand, continues to show circadian oscillations of electrical activity.[61] If the SCN is surgically removed from the brain and studied *in vitro*, circadian rhythms of 2-deoxyglucose

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Abbreviations: 2-DG, 2-deoxyglucose; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EPSP, excitatory postsynaptic potential; GRP, gastrin releasing peptide; IPSP, inhibitory postsynaptic potential; NAAG, *N*-acetyl aspartyl glutamate; NMDA, *N*-methyl-D-aspartate; SCN, suprachiasmatic nucleus; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide.

(2-DG) utilization[145] and peptide release[37] can still be found. Transplantation of a neonatal SCN into an adult rodent whose own SCN has been destroyed results in a rapid recovery of the host's behavioral circadian rhythms.[80,121]

In humans, the biological clock governing circadian rhythms plays a critical role in jet lag, work shift performance, sleep–wake cycles, and manifestation of certain psychiatric disorders. The human SCN contains most of the same neuroactive substances found in rodents, and in many respects shows a parallel cytochemical organization.[88,98,150] The presence of a direct retinal projection to the SCN in other mammals, including non-human primates, suggests that a homologous projection exists in humans.[98] Humans suffering from senile dementia may have problems with their circadian rhythm and have been reported to show a deterioration in the SCN, as evidenced by a decrease in cell number.[96,158] Tumors near the hypothalamus that compress the SCN have been correlated with loss of circadian rhythmicity in humans.[98] Taken together, these findings suggest that the human SCN may function in a manner parallel to laboratory animals, and that modes of interaction between cells in the SCN of other animals can be used to predict how similar cells in the human SCN may communicate.

Electrophysiological evidence from single- and multiple-unit recordings in hypothalamic slices containing the SCN has documented a circadian rhythm of neuronal activity, at least at a population level.[13,47,147] Converging lines of anatomical,[99,166] cytochemical[20,173] and electrophysiological[11,71] evidence have demonstrated that the SCN is composed of a heterogeneous population of neurons. That several thousand individual cellular oscillators in the SCN could stay entrained in the absence of intercellular communication is highly unlikely. Instead, it seems that some form of intercellular communication is required to maintain a circadian rhythm of electrical activity in the SCN.

The purpose of our paper is to examine some of the unusual attributes of the SCN, and to consider possible modes of intercellular communication which may operate here and may explain some of the unexpected findings concerning the function of the SCN as a circadian clock. The physiological mechanism whereby the SCN measures time in the absence of environmental cues is not known. Intercellular communication may play a crucial role in the generation of circadian time, or may provide a means of averaging individual clock cells that have an inaccurate circadian rhythm[11] to generate a more accurate

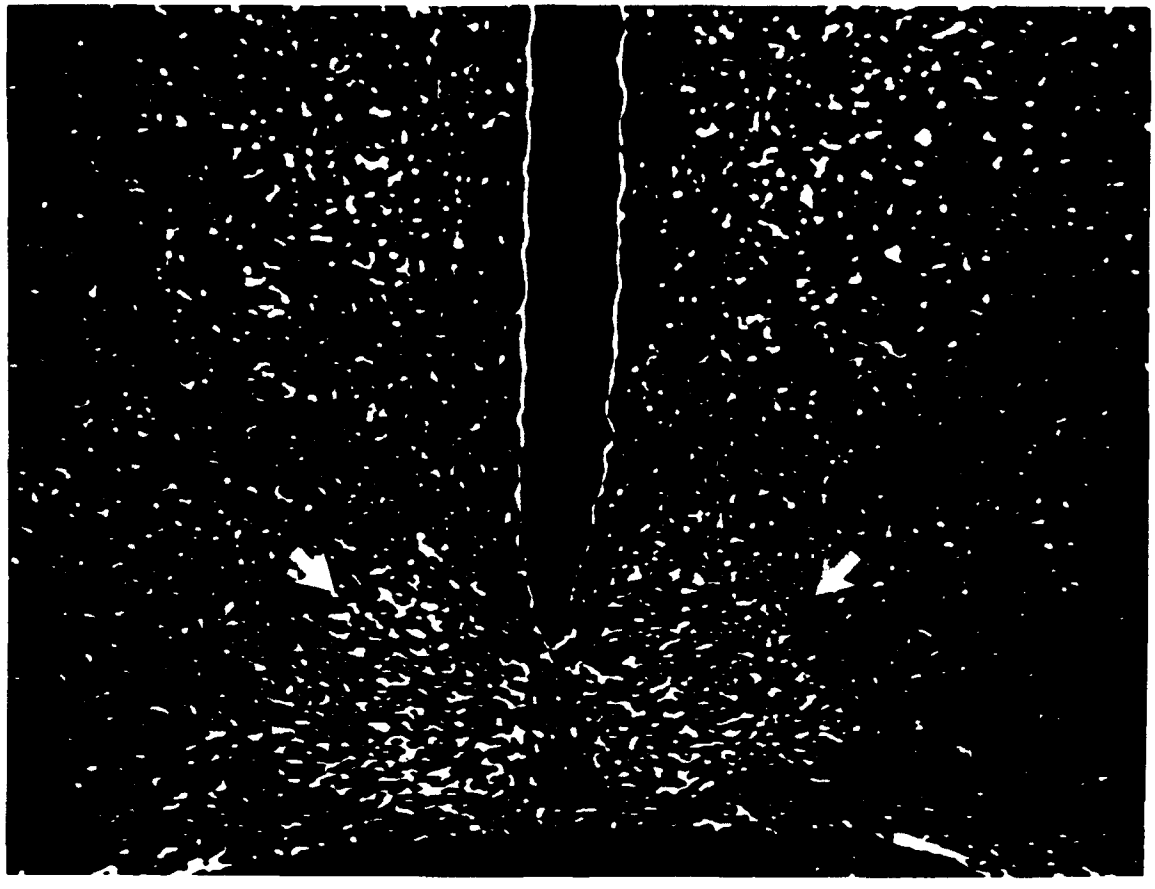

Fig. 1. The small, densely packed Nissl-stained cells of the SCN (arrows) are shown on either side of the third ventricle in mouse hypothalamus. Width of micrograph: 1.1 mm.

24-h clock. Certainly, the ability of the master circadian oscillator to drive the rest of the brain is dependent on some efferent signal. This paper will address two main issues: (i) How do SCN cells receive and coordinate their temporal information within the SCN? and (ii) How do SCN cells impose their circadian message on other neurons in the CNS?

The bilateral SCN is composed of about 20,000 cells that have typical electrophysiological events such as synaptic potentials (Fig. 2) and action potentials (Figs 3, 4). These high frequency events operating in the millisecond time scale are somehow integrated to provide an oscillator with a 24-h period (Fig. 3A–F). The SCN is remarkably accurate in its ability to generate a rhythm with a free-running period of close to 24 h in length, a cellularly generated rhythm which may continue to oscillate accurately for years in the absence of external cues.[127]

2. RETINAL INPUT TO SUPRACHIASMATIC NUCLEUS

The diurnal light–dark cycle entrains the circadian rhythm via afferent connections from the visual system to the hypothalamus. The axonal projection from retinal ganglion cells to the SCN has been demonstrated by anatomical methods.[55,101] The detection of the retino-suprachiasmatic pathway provided one of the first clues regarding the location of the mammalian circadian clock. Early studies indicated that most SCN cells were excited by retinal or optic nerve stimulation.[48,49,89,93,108,131,146] The best transmitter candidate for the retino-SCN projection is an excitatory amino acid, probably glutamate. This is supported by several findings: glutamate is released from stimulated optic nerves;[81] glutamate immunoreactivity is present in presynaptic terminals in the SCN;[168] administration of glutamate to the SCN causes phase shifts;[91] applications of glutamate antagonists block the light-induced phase shifts;[26] SCN cells respond to glutamate with an increase in electrical activity[107] and an increase in intracellular calcium;[169] and broad-spectrum glutamate antagonists block extracellularly recorded postsynaptic responses to optic nerve stimulation in the SCN.[18,19,143] Intracellular recordings in the SCN have shown that non-*N*-methyl-D-aspartate (NMDA) glutamate antagonists block retinally evoked excitatory postsynaptic potentials (EPSPs) at or below resting potential (Fig. 2A). NMDA antagonists block or reduce these EPSPs when the cells are depolarized, indicating that both the NMDA and non-NMDA types of receptors contribute to retinally evoked excitation and that the contribution of the different receptors depends on the postsynaptic membrane potential.[69,71] The combination of NMDA and non-NMDA glutamate antagonists blocks virtually all of the response of postsynaptic cells to optic nerve stimulation, indicating that an excitatory amino acid, probably glutamate, is necessary for retinal axon excitation of the SCN. Together, these data demonstrate that the main afferent input to the SCN, which plays a critical role in resetting the circadian clock, depends on optic nerve axonal action potentials that cause release of an excitatory amino acid transmitter.

As at least some of the retinal input to SCN may be provided by axon collaterals projecting to other visual regions,[116] the identity of the transmitter in these other regions serves to elucidate further the identity of the retino-SCN transmitter. Glutamate appears to serve as the primary retinal transmitter in the lateral geniculate nucleus in the thalamus[133,148] and the superior colliculus.[57] Glutamate also plays an important role in the afferent regulation not only of the SCN, but also of other regions of the medial hypothalamus adjacent to the SCN, some of which receive SCN axonal input.[174] Glutamate is an intrinsic excitatory transmitter released by many medial hypothalamic neurons.[172]

Further support for glutamate as the primary optic nerve transmitter to the SCN is provided by recent findings which suggest that expression of the early immediate gene cFOS and related genes in the SCN induced by light presented during the dark part of the environmental light cycle[76,123,129] can be blocked by the intraperitoneal administration of glutamate antagonists.[1,2] The dipeptide *N*-acetyl aspartyl gluta-

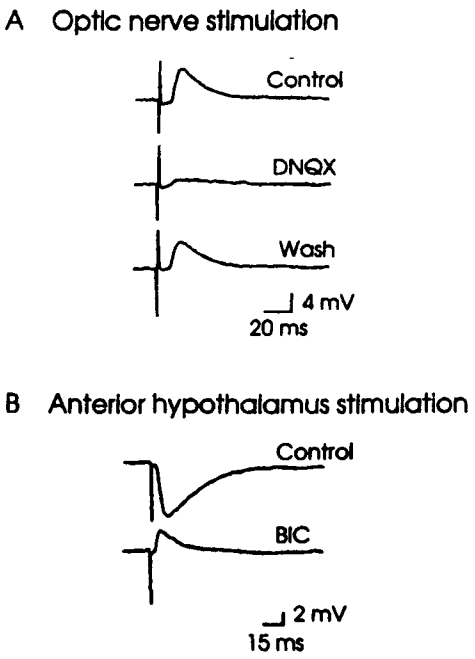

Fig. 2. Excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) recorded intracellularly from SCN neurons in parasagittal hypothalamic slices. (A) EPSPs evoked by stimulation of the optic nerve in normal solution (Control) are blocked by bath application of the non-NMDA receptor antagonist 6,7-dinitroquinoxaline-2,3 dione (DNQX, 10 μm). This block is reversible (Wash). From Kim and Dudek.[69] (B) IPSPs evoked by stimulation of the medial hypothalamus (dorsocaudal to the SCN, Control) are blocked by the GABA antagonist bicuculline methiodide (BIC, 20 μm). This block occasionally revealed EPSPs. Neither DNQX nor bicuculline altered input resistances. From Kim and Dudek.[70]

mate (NAAG) has been detected in retinal axons, raising the possibility that it, too, may serve as a retinal transmitter in the SCN.[97] There is little direct evidence that NAAG has a significant effect on SCN neurons. NAAG appears to activate specifically the NMDA-type glutamate receptor.[164] The non-NMDA receptor antagonist 6,7-dinitro-quinoxaline-2,3-dione (DNQX) blocks a large part of the response of SCN cells to optic nerve stimulation in hypothalamic slices,[69,71] although a greater contribution of NMDA receptors might be expected *in vivo* if a higher level of cellular activity is present and neurons are more depolarized. None the less, it appears unlikely that NAAG functions as the crucial neurotransmitter in the retino-SCN pathway, although it may be co-released with glutamate.

The SCN receives another input from the visual system indirectly via the intergeniculate leaflet. The fibers terminating in the SCN from the intergeniculate leaflet are immunoreactive for neuropeptide Y,[21,54] and some may contain GABA.[23] The SCN receives input from a number of other regions of the brain, including a large serotonin projection from the raphe[3] and GABAergic input from the surrounding hypothalamus.[70] Further discussion of SCN afferent axons can be found elsewhere.[73]

3. LOCAL CIRCUITS AND SUPRACHIASMATIC NUCLEUS NEUROTRANSMITTERS

The axons of many SCN neurons terminate within the nucleus; these could be either local circuit axons, or collaterals of longer projection axons.[166] The inhibitory amino acid transmitter GABA is found in half of all presynaptic terminals in the SCN.[32] GABA, its synthetic enzyme glutamate decarboxylase, and mRNA coding for glutamate decarboxylase are found in most SCN neuronal cell bodies.[22,113,161,173] The presence of GABA in most SCN cells suggests that the predominant effect that SCN axons exert on other cells is inhibitory. Electrical stimulation of the medial hypothalamus near the SCN consistently evokes inhibitory postsynaptic potentials (IPSPs) in SCN neurons (Fig. 2B), and spontaneous IPSPs are commonly found in slice preparations of the SCN; these IPSPs appear to be mediated by $GABA_A$ receptors regulating increases in chloride conductance.[70] Further support for an inhibitory role for SCN neurons comes from experiments where SCN neurons were consistently depressed after SCN efferent projections to the periventricular area of the hypothalamus were antidromically stimulated;[77] this depression could be due to GABA-mediated inhibition from local collaterals of antidromically stimulated projection axons. A few reports have suggested that stimulation of the retinal projection to the SCN can elicit inhibitory responses in SCN neurons,[90] but these may be due to retinal excitation of local inhibitory circuits. Thus, on the basis of both physiological and immunocytochemical grounds, the inhibitory transmitter GABA appears to be the major known transmitter of the SCN.

In addition to GABA, SCN neurons synthesize several other neuroactive substances that may function as neurotransmitters. These include the peptides vasopressin,[151,175] vasoactive intestinal polypeptide (VIP),[20] gastrin releasing peptide (GRP),[128,173] peptide histidine isoleucine, somatostatin, and neurotensin. Immunoreactivity for many of these peptides has been detected in axon endings in synaptic contact with SCN cells.[167] Studies employing dual ultrastructural markers to stain two neurotransmitter antigens or uptake systems simultaneously have determined the neurochemical identity of both pre- and postsynaptic neurons. As for substances which may relate to local circuit interactions, GABA-immunoreactive axons terminate on GABA- and GRP-immunoreactive cells, GRP- and vasopressin-immunoreactive axons terminate, respectively, on cells showing the same immunoreactivity, single cells receive input from both GABA and GRP or GABA and VIP,[170] and somatostatin-immunoreactive axons terminate on VIP-immunoreactive perikarya.[87] Other axons likely to originate outside the SCN contain neuropeptide Y and serotonin immunoreactivity and terminate on the same postsynaptic SCN cell.[51] Similarly, neuropeptide Y-[181] or serotonin-[59,72] immunoreactive fibers terminate on VIP-immunoreactive SCN cells. These data suggest a complex neurochemical synaptology within the SCN. Combinations of SCN transmitters may show a synergistic action on behavioral phase-shifts and electrical activity; a combination of such neuroactive substances (VIP + GRP + peptide histidine isoleucine) generates a stronger response than any of the substances separately or in pairs.[4]

Since GABA is found in most of the cell bodies and in half the terminals, it probably co-exists intracellularly with most of the peptides described in the SCN, and may be co-released with them. This is further supported by the consistent presence of dense-core vesicles in GABA- as well as in glutamate-immunoreactive terminals in the SCN area.[32,168] Although release of amino acid transmitters is generally considered to operate via a calcium-mediated mechanism at the axon terminal, in some areas of the nervous system this may not be the case. For instance, retinal bipolar cells may release GABA in a calcium-independent manner, perhaps by a plasmalemmal GABA transporter.[6,135,182] Although no data have been found in support of similar release from SCN neurons, such a release mechanism may serve to mediate local circuit interactions and might operate independently of neural firing.

4. CELLULAR INTERACTION AND THEORETICAL MODELS OF BIOLOGICAL CLOCKS

Local cell communication may play a critical role in the synthesis of a circadian oscillation and/or in

increasing the stability of the circadian period. Ultradian oscillations of intracellular calcium occur in SCN neurons and astrocytes in culture.[169] Similarly, action potentials with regular (Fig. 3A, top) and irregular (Fig. 3A, bottom) periodicity are found in the SCN in slice preparations, and this periodicity can be modified by the level of depolarization.[71] Mathematical models have suggested that an aggregate rhythm can be faster or slower than any individual oscillator in the group and that coupling can result in an aggregate rhythm with a more precise oscillation.[180] Along these lines, individual SCN neurons show cycles of activity with periods of 24 ± 6 h.[11] Mathematical models have suggested that a group of high frequency oscillators may serve as a basis for a low frequency oscillator such as a circadian clock (see Fig. 3).[114]

That oscillators with a period of a few minutes could be used to build a circadian clock[114,134] is consistent with some of the periods found in SCN cells with calcium digital imaging. In SCN neurons (Fig. 3B) and astrocytes (Fig. 3C–E), ultradian oscillations with periods of 8–20 s can be initiated or altered by GABA, glutamate, and serotonin, transmitters found in large numbers of axon terminals in the SCN.[169] The localization of the inhibitory transmitter GABA in most SCN neurons may provide support for a theoretical model of circadian clocks,[134] where an ensemble of self-oscillators all operate to inhibit each other, providing a mechanism both for phase-shifting and for long-term clock stability. Continuous release of GABA may provide a means of regulating other cells. For instance, tonic release of GABA in the retina may regulate release of some catecholamines; no transmitters have been identified that induce release of dopamine from fish interplexiform cells,[34] whereas application of GABA antagonists does induce dopamine release,[112] suggesting a constant inhibition of dopamine release by GABA.

Specific testable theoretical models of circadian operation based on structural and electrophysiological data from the SCN have not yet been generated.

5. CELLULAR COMMUNICATION WITHIN THE SUPRACHIASMATIC NUCLEUS

The most commonly encountered method of neuronal communication in the brain involves sodium-dependent action potentials travelling along the axons, and calcium-dependent release of neurotransmitter from the axon terminal at the synapse;[15,29,41,64] sodium-dependent action potentials are common in the SCN (Fig. 4A). Recent experiments which blocked the sodium-mediated action potentials in the SCN suggest that other mechanisms synchronize the activity of neurons in this nucleus.

One of the first indications that the circadian clock can keep time without sending efferent messages to other regions of the brain came from early experiments showing that electroconvulsive shock and exposure to nitrous oxide blocked expression of the circadian clock for up to 10 days. When the animals again began showing activity cycles, they were initiated at exactly the point that would have been predicted if the clock had continued to keep time while not sending temporal information to other parts of the brain.[127] More recently, the specific role of Na^+-dependent action potentials in circadian rhythms was evaluated by injecting tetrodotoxin (TTX) directly into the SCN *in vivo* to block action potentials and synaptic potentials. TTX blocked circadian rhythms of behavioral activity.[90,136,139] Similarly, TTX blocked entrainment to the environmental light–dark cycle. When TTX administration was halted, however, the animals resumed their activity cycle at precisely the phase of the free-running cycle that would have been predicted if the endogenous circadian clock had continued to operate. During the time of TTX administration, light stimulation had no immediate or latent effect on the circadian rhythm. Similar results were obtained *in vitro* where circadian rhythms of vasopressin release were blocked by TTX, but recovered upon TTX washout.[37] Thus, TTX blocks the manifestation of the clock, probably by inactivating efferent axons, and it blocks the ability of the clock to entrain to environmental light, probably by inactivating afferent retinal axons, but it does not interfere with the clock mechanism itself. A critical additional experiment will be to record from individual SCN cells during administration of TTX at concentrations high enough to block rhythm expression, in order to demonstrate that all sodium spikes are fully blocked and to determine whether calcium spikes are present.

TTX generally blocks axonal action potentials by blocking voltage-dependent sodium channels,[58] effectively isolating the synaptic input at the cell body and dendrites from the electrotonically distant neurotransmitter release sites in the axon terminals, but does not block spontaneous release of neurotransmitter from single synaptic vesicles (i.e. quantal events detected as miniature synaptic currents).[38,65] TTX-insensitive sodium channels which can spike in the presence of TTX have been characterized in other regions, including neurons of the rat nodose ganglion,[60] cranial sensory neurons,[12] developing muscle cells,[44] and frog dorsal root ganglion neurons,[103] but have not been detected in the SCN. TTX blocks the optic nerve input to SCN neurons.[146] The cell bodies of SCN neurons, and presumably their axons, have TTX-sensitive Na^+ channels;[157] TTX is reported to block spontaneous and induced SCN action potentials *in vitro*.[176] These *in vitro* electrophysiological experiments provide support for the interpretation that the TTX treatment employed to block the expression of circadian time[37,139] blocked the sodium channel-mediated action potentials of afferent axons from the retina, of axons and dendrites within the SCN, and of efferent axons from the SCN.

An interesting parallel has been reported within the retina itself. Ganglion cells, several synapses away from the photoreceptors, still responded to light even when the retina was bathed in TTX.[104]

Block and recovery of circadian rhythms similar to the TTX experiments have been reported for SCN injections of procaine.[136] Procaine is a local anesthetic, and it blocks action potentials by depressing voltage-dependent sodium channels. Like other local anesthetics, the mechanism is frequency dependent, so that procaine blocks trains of action potential more effectively than single action potentials.[28]

These results obtained by the use of TTX and procaine suggest that both the input to the clock from the retina and the output of the clock are dependent on the commonly encountered mechanisms of axonal conduction and transmitter release, but intercellular communication within the SCN can operate by some other means. What alternate mechanisms of communication inside the biological clock might allow the cellular elements of the clock to co-ordinate their function?

5.1. *Calcium spikes*

Although sodium spikes are blocked by TTX, calcium spikes are not.[84] Since calcium spikes have been found in SCN slices[157] (Fig. 4B), they merit consideration for a possible role in transmitter release and interneuronal communication. Whereas there is little evidence that axons conduct propagated Ca^{2+} spikes, it is widely thought that dendrites can actively do so.[58,82] It is unlikely that Ca^{2+} spikes could account for presynaptic release of transmitters at the terminal regions of long axons, but they may play a role in dendritic transmitter release or release from axons close to the perikaryon. Such SCN axons that terminate near the cell body of origin have been found with Golgi silver chromate impregnations.[166]

As calcium spikes are different from sodium spikes in several respects, a brief overview may be useful for

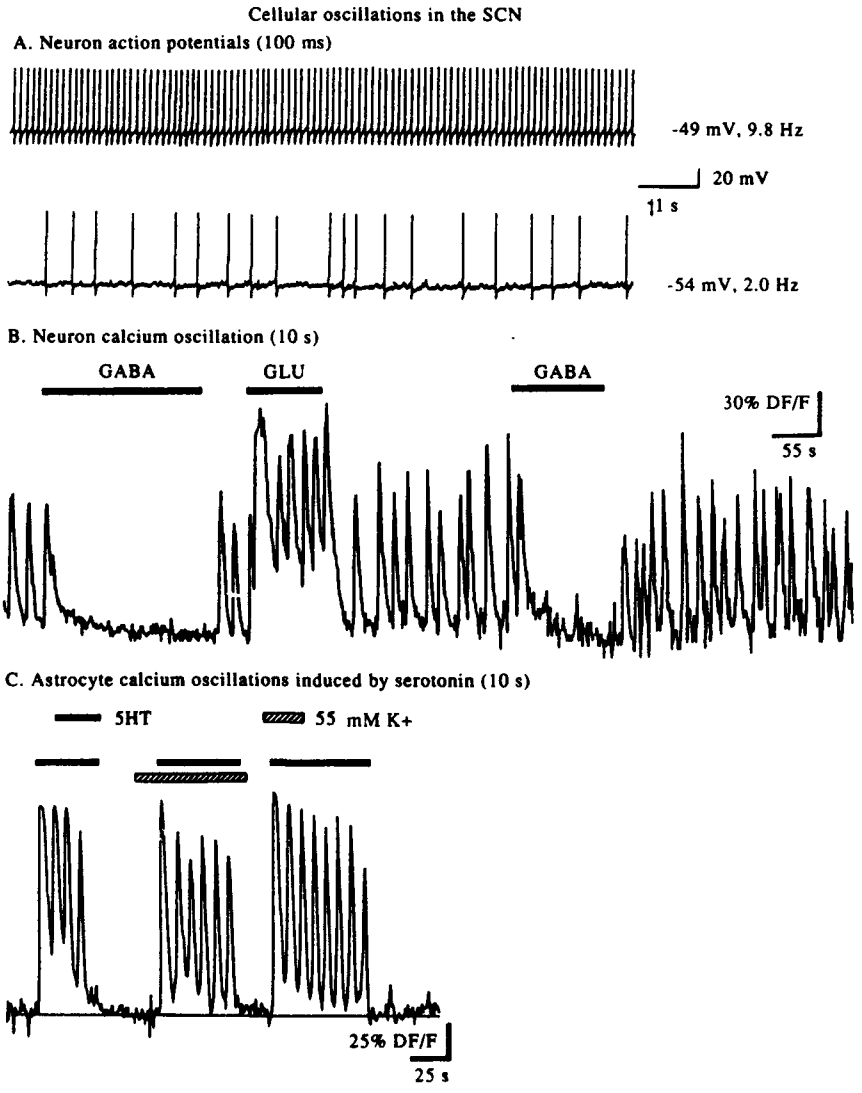

Fig. 3 A–C

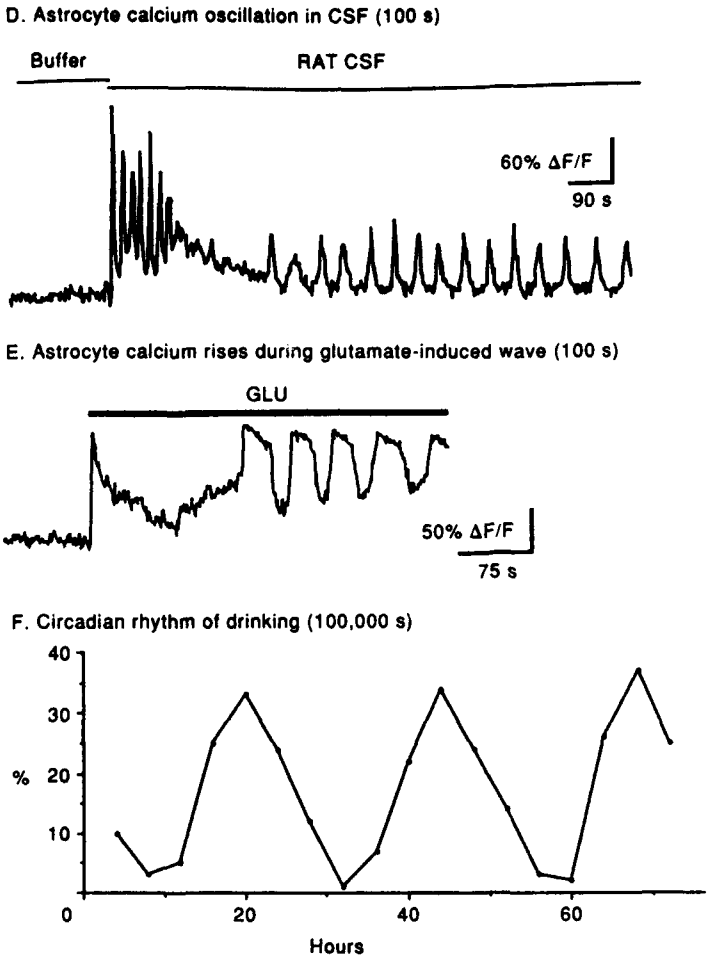

Fig. 3. Cellular oscillations in the SCN. Some examples of regular SCN oscillations are shown here, with the approximate period of the oscillation in parentheses. (A) Action potentials with a regular period of 100 ms can be found in neurons in SCN slices; the interspike interval can be changed by increasing the membrane potential as seen in the bottom part. From Kim and Dudek.[71] (B) Calcium oscillations with a period of about 20 s in SCN neurons can be influenced by glutamate, which increases the baseline calcium level here, and by GABA, which blocks expression of the calcium oscillation. (C) In an SCN astrocyte, calcium oscillations with a period of about 10 s are initiated by perfusion of serotonin. The oscillation in this cell was not due to depolarization because high levels of potassium without serotonin had little effect. (D) In rat cerebrospinal fluid, an astrocyte exhibits a regular oscillation of intracellular calcium which changes from a fast to a slow oscillation after about 200 s. (E) Glutamate induces calcium waves which move from astrocyte to astrocyte. Calcium rises with a period of about 55 s are due to the astrocyte's involvement in periodic intercellular waves. B–E are from van den Pol *et al.*[69] (F) Endogenous circadian rhythm of drinking behaviour in rats over a three-day period (72 h). The ordinate shows the relative percentage of drinking that occurred in a day for that particular 4-h time bin. This trace shows the mean drinking behavior, measured electronically, in several rats in the absence of light cues.

understanding possible roles of calcium spikes in intercellular communication. Two types of calcium spikes, high- and low-threshold, have been suggested in SCN neurons.[69,157,163] These types of calcium spikes have been described in other parts of the CNS, including other regions of the hypothalamus, thalamus, and neocortex.[82,95] High-threshold calcium spikes were first recognized in barnacle muscle fiber and in gastropod giant neurons,[43,52] and have been found in mammalian cerebellar Purkinje cells and hippocampal pyramidal cells, where they were attributed to calcium channels in dendrites. When sodium spikes are blocked and a cell is depolarized, a long-duration high-threshold action potential can be evoked; this high-threshold calcium spike is eliminated by low-calcium solutions or calcium channel blockers. Excitatory synaptic events trigger sodium spikes, and the tail-end of these sodium spikes has a high-threshold calcium component. Low-threshold calcium spikes have been described in inferior olivary cells.[82,85] The low-threshold calcium current is different from the high-threshold current, because this mechanism is inactivated at resting potential in many cells and deinactivated when the cell is hyperpolarized. Thus, in a hyperpolarized cell, a brief depolarization, such as a synaptic event, induces a

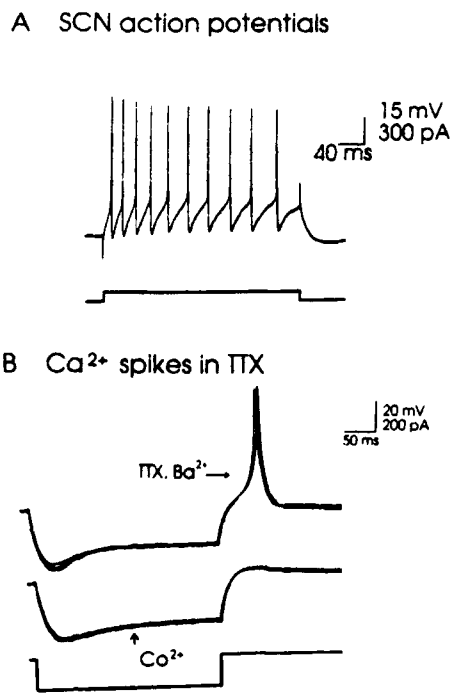

Fig. 4. Action potentials recorded intracellularly from SCN neurons. (A) In normal solution, a depolarizing current pulse evokes a train of action potentials. These spikes showed a slight, progressive decline in amplitude and frequency during the spike train, which is typical for most hypothalamic neurons. From Kim and Dudek.[71] (B) To study calcium (Ca^{2+}) spikes in SCN neurons, TTX was used in all buffer solutions to block sodium spikes. Barium (Ba^{2+}), which produces a greater current through calcium channels than calcium itself, was substituted for calcium. The rebound depolarization to a hyperpolarizing current pulse evoked a long-duration calcium spike (horizontal arrow). When cobalt (Co^{2+}), which blocks the calcium channel, was substituted for barium. Adapted from Sugimori *et al.*[157]

low-threshold calcium spike that has superimposed sodium spikes. Therefore, either low- or high-threshold calcium spikes could be initiated in the presence of TTX, propagate along SCN dendrites, and cause secretion of a neuroactive substance from release sites in dendrites, and therefore could play a role in intercellular communication in the SCN.

That calcium may be functionally important to circadian rhythms has been shown in several systems. For instance, calcium is involved in the *Bulla* pacemaker, where it participates in phase control.[66,67] Reducing extracellular calcium has been reported to block circadian rhythms of the SCN *in vitro*.[145]

5.2. *Presynaptic dendrites*

Presynaptic dendrites have been described in the SCN.[50] In comparison with regions such as the olfactory bulb, however, where presynaptic dendrites of granule and mitral cells participate in the majority of synaptic interactions,[141] the number of presynaptic dendrites in the SCN is very small. Immunostaining for neuroactive substances in the SCN shows them to be localized most strongly in axons, less in cell bodies and proximal dendrites, and hardly at all in distal dendrites. Despite the relative lack of evidence for the presence of transmitters in SCN dendrites, the possibility of dendritic release of a neuroactive substance cannot be completely ruled out. However, given the relative rarity of presynaptic dendrites in the SCN, coupling presynaptic dendrites with dendritic calcium spikes could account for only a small fraction of normal intercellular communication in the SCN.

5.3. *Paracrine–hormonal interaction*

A closely related possibility is that calcium action potentials mediate "paracrine" or hormonal release of a neuroactive substance from axonal or dendritic regions that are not presynaptic. That dendrites can have release mechanisms has been demonstrated in the substantia nigra, where the transmitter dopamine[106] and the enzyme acetylcholinesterase[83] are released from dendrites at non-synaptic areas of the membrane. At present, there is no direct evidence for this in the SCN. Given the high number of local axon collaterals, it will be difficult to test the non-synaptic dendritic release hypothesis in the SCN.

5.4. *Graded potentials*

In invertebrates, particularly in some locust interneurons,[17,79] in the lobster stomatogastric ganglion[46,140] and in some areas of the mammalian brain such as the olfactory bulb[141] and retina,[14] neurons may release a transmitter as a result of changes in local membrane potential, independent of propagated potentials. An example of this is the axon-less olfactory bulb granule cell, which releases GABA from presynaptic dendrites in response to local excitatory stimulation arising from mitral cell presynaptic dendrites. Some cells in the retina have been postulated to communicate with other neurons with mechanisms mediated only by graded potentials and not by action potentials. A disadvantage of studying graded potentials in the SCN relative to the retina and olfactory bulb is the clear laminar structure and ease of identification of different cell types in the latter two areas, but not in the SCN. Although many of the neurons in the SCN have identifiable axons based on Golgi impregnations[166] and dye injections, cells without axons are sometimes seen. The absence of detectable axons in these cells has been assumed to be a result of incomplete Golgi impregnations, incomplete filling, or the very small diameter of axons making them difficult to visualize with the light microscope. On the other hand, it is not out of the question that some of these cells may not have axons. Graded potentials in small SCN neurons could spread short distances according to the cable properties of the dendrite or short axon, but would not travel over long distances.

5.5. *Field effects*

The possibility that electrical field effects play a role in intercellular signalling within the SCN appears

unlikely. The close apposition of neuronal membranes without intervening glia combined with the small volume of extracellular space and presumed high extracellular resistance would tend to enhance the amplitude of extracellular field potentials. The classical case for electrical field effects is the Mauthner cell system of the goldfish.[40] In the mammalian brain, evidence for electrical field effects has been found in the hippocampus.[16] Two basic principles concerning field effects have emerged from earlier data. First, close apposition of membranes and small extracellular space with high extracellular resistance are important for enhancing electrical field effects. Second, having the neurons organized in a laminar fashion is equally critical, because this effectively makes the extracellular currents summate. In the SCN, although the extracellular space is small, the neurons are not arranged in a laminar fashion; instead, the dendritic arbors are randomly oriented[166] and their electrical fields would be expected to cancel each other rather than summate. The field potentials previously observed in the SCN after synchronous activation of optic nerve fibers were only tens of microvolts;[19] experiments in the hippocampus suggest that transmembrane voltage changes are generally about 50% of the field potential amplitude,[36,162] thus suggesting that in the SCN electrical field effects would be relatively small, in the range of several microvolts, and they would not be expected to contribute significantly to neuronal communication.

5.6. *Gap junctions*

Gap junctions may play an important role in cellular communication in other biological clocks. Fragments of pineal tissue show rhythms of melatonin secretion,[160] and gap junctions have been found between these cells. In the marine mollusks *Bulla* and *Aplysia*, the eye shows circadian rhythms of activity; these cells are also coupled by gap junctions.[62,63] In addition to electrotonic coupling, gap junctions may be important in other types of chemical signalling. Because gap junctions allow transmission of chemical signals with molecular weights smaller than 1000 mol. wt, a variety of intercellular messengers could pass through these structures, thereby synchronizing the activity of coupled cells.[117,152] For example, several lines of evidence suggest that small molecules such as cAMP and inositol triphosphate may cross gap junctions as a means of distributing cellular signals.[142] In light of this, it is interesting that phase changes in the electrical activity of SCN slices *in vitro* can be induced by cAMP analogs[118] and that cAMP shows a circadian variation in SCN slices held under constant conditions.[119]

Although credible evidence for gap junctions or electrotonic coupling between neurons has not been reported in the SCN, large gap junctions are common between astroglia throughout the SCN.[166] Gap junctions between neurons in the mammalian brain are very rare, and have been reported in only a handful of structures in all species examined.[115] In recent years, the proteins, called connexins, that make up gap junctions have been isolated and characterized. As new probes and antibodies for the connexin gap junction proteins become available, it will be of interest to search for their presence between SCN neurons.

5.7. *Ionic interactions*

Another possibility for neuronal communication is by non-synaptic ionic means. Because the circadian rhythm of electrical activity in the SCN is essentially a cell population phenomenon, it is possible that periods of increased electrical activity are associated with increases in extracellular potassium concentration and decreases in extracellular calcium. In the SCN, and particularly in the dorsomedial region of the rodent SCN, the cell bodies of the neurons are closely apposed without intervening glial cells. Regions of apposition may vary from several square micrometers to over 85 μm^2; some cells are held together by a specialized puncta adherens. Sometimes many cells, mostly neurons, are lined up adjacent to one another in a rostrocaudal direction with no astrocyte insulation.[166] The short distance between adjacent cells and the extremely small volume of extracellular space would tend to maximize the influence of any ions released by one cell on adjacent cells. For instance, during electrical activity, the concentration of extracellular potassium tends to rise. Even a small change in this ion would lead to an increase in the excitability of an adjacent neuron. Just as a single synapse may not induce an action potential in the postsynaptic cell, ionic interaction may not lead to a one-to-one response, but may simply influence the "probability" of firing of adjacent neurons. The additional effect of activity-related decreases in extracellular calcium would also influence adjacent neurons.

High dietary potassium has been reported to cause a phase advance in circadian rhythms,[74] and infusion of high levels of potassium into the SCN are reported to cause a phase shift in the circadian clock.[136] These general alterations in ionic balances would influence many aspects of intercellular communication and therefore it is difficult to define the specific cellular substrate underlying the effects on circadian rhythms. Studies altering the extracellular ionic milieu of the SCN *in vitro*, or using potassium-sensitive electrodes to monitor extracellular potassium in the intact SCN, should facilitate our understanding of the role of ionic mechanisms in inducing phase-shifts and influencing the period of the clock cycle.

Recent studies on hypothalamic slices bathed in calcium-free medium have indicated that bursts of action potentials can be synchronized in the SCN by mechanisms that do not involve chemical synaptic transmission.[13] Dual multi-unit recordings in calcium-free solution containing a mixture of glutamate and $GABA_A$ antagonists showed that two

groups of neurons in the same SCN had synchronized bursts of activity (Fig. 5). That the experimental conditions were sufficient to block synaptic transmission was seen by the absence of EPSPs in whole-cell, patch-clamp recordings under these conditions. These data provide evidence that SCN neurons can be synchronized without detectable active chemical synapses, and that another mechanism of communication can underlie synchronized activity.

5.8. *Glial communication*

Although it has generally been assumed that neurons are the site of the circadian clock, there is not yet any definitive evidence that the SCN clock is neuronal. Since the ionic homeostasis of the extracellular space is regulated by glial cells, changes in the glial regulation of extracellular ions, particularly potassium and calcium, could be a critical factor regulating the circadian rhythm. If glial cells are involved in regulating rhythms, they would need to be receptive to photic information from the eye. SCN astrocytes do show a dramatic rise in intracellular calcium when stimulated with glutamate. An interesting parallel is found in the retina where glial cells (Müller cells) show an electrical response to light, probably via K^+ released by nearby neurons.[94] This retinal glial cell gives rise to the B wave of the electroretinogram.

Very regular periods of intracellular calcium oscillations are seen in astrocytes; these fluctuations of calcium can be induced by glutamate, glutamate agonists and serotonin, and have a period of 8–20 s in cultured SCN cells (Fig. 3C). Similarly, astrocytes in SCN organotypic cultures exhibit calcium oscillations in the presence of rat cerebrospinal fluid. Periodic waves of calcium with a period of 30 s to 2 min move from glial cell to glial cell after neurotransmitter stimulation *in vitro* (Fig. 3E).[169] This intercellular communication between astrocytes is probably based on gap junctions, allowing calcium, inositol triphosphate or some related message through the gap junction.[10,24] Long-distance communication between astrocytes is not restricted to the SCN, but has also been found between hippocampal cells *in vitro*.[27] That this intercellular communication is not simply an artifact of culturing is substantiated by the recent finding that electrical stimulation of glutamatergic axons in slices of hippocampus activates an astrocyte response in the region of the axon terminals.[30] Calcium fluxes and intercellular astrocyte communication in the SCN can also be induced by serotonin, another transmitter in afferent axons to the SCN from the raphe, and by extracellular ATP.[169]

SCN astrocytes exhibit a heterogeneous response to different transmitters. Of the agents we have tested, some astrocytes respond with a calcium increase only to glutamate or serotonin, some respond to both glutamate and serotonin, and some respond to glutamate, serotonin, and ATP. Astrocytes from the SCN appear different from astrocytes from the surrounding hypothalamus in that the SCN cells show a much stronger level of immunostaining for glial fibrillary acidic protein.[102]

If glial cells play a role in modulating or driving circadian rhythms, they must, in some manner, influence neuronal excitability. They may regulate extracellular ionic levels, particularly potassium or calcium, by their reponse to neurotransmitters.[14,110,120,165] Alternatively, glial cells may release some substance, for instance arachidonic acid,[5,16] which has been shown to reduce glutamate uptake from extracellular space.[8] An increase in extracellular glutamate would alter the excitability of SCN neurons.[169]

Whereas the circadian rhythms are probably generated by neurons, the glial hypothesis merits some consideration. In *Drosophila* the *per* gene is necessary for expression of circadian rhythms;[75] subsets of both neurons and glia express *per*.[184] Fly genetic mosaics which did not express *per* showed no circadian rhythm, whereas flies that showed *per* expression only in glial cells did show a circadian rhythm.[39] The most robust circadian rhythm was found in flies in which both neurons and glial cells expressed *per*. In experiments examining circadian rhythms in rodents, one could try to destroy either the astrocytes or the neurons. However, even if a selective and effective means of killing only glial cells could be found, and this resulted in a loss of circadian rhythms, this could actually be due to severely compromised neurons given the important sustaining roles glial cells play in neuronal survival.[7] On the other hand, neurons could potentially be selectively destroyed, for instance by administration of high levels of glutamate which would selectively kill neurons but not glial cells.[42] A problem with this type of experiment is that even if the glial cells are the "clock" cells, the efferent message to other parts of the brain is probably conveyed by neuronal axons, as it can be blocked by TTX. These problems of interpretation may make it difficult to test the relative contribution of glial cells and neurons to circadian rhythms. Transplantation may offer another approach to the glial/neuron question if viable purified populations of glia or neurons were isolated from the SCN. This would only be feasible if the cellular components of the clock would reaggregate in a phase-related functional manner, and be able to send their efferent message to other brain loci.

6. INTERCELLULAR COMMUNICATION IN EARLY DEVELOPMENT

Several lines of evidence suggest that the cells of the SCN may demonstrate orchestrated circadian rhythms at early stages of development. Of considerable interest to the question of intercellular communication within the SCN are experiments showing that even before the time of major synapse formation (i.e. before birth in rats), radiolabeled 2-DG uptake in

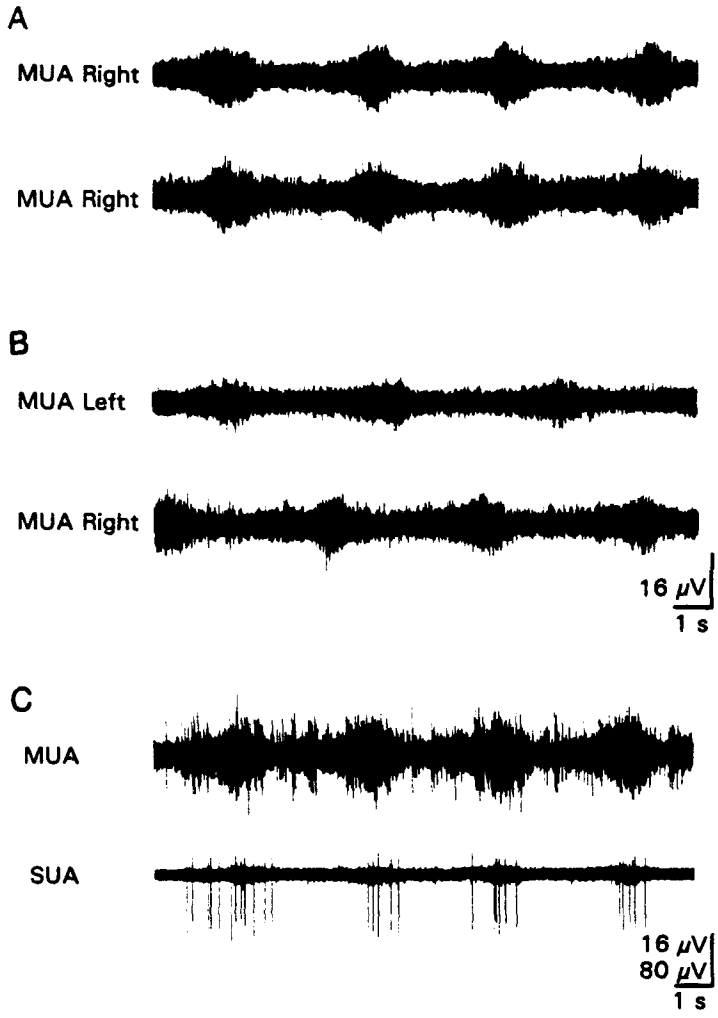

Fig. 5. Simultaneous recordings of extracellular spike bursts in Ca^{2+}-free medium containing BAPTA. (A) Simultaneous extracellular recordings of multiple-unit spike activity from two sites separated by 150 μm in one SCN (right side) demonstrated synchronous bursts of action potentials. (B) Similar recordings of multiple-unit activity from left and right SCNs showed that although the bursts were present on both sides, they were not synchronous and their interburst intervals were different (same slice as A). (C) Single-unit recording during a burst of multiple-unit activity in an SCN. The bursts of single-unit activity coincided with the bursts of multiple-unit activity. From Bouskila and Dudek.[13]

SCN cells appears to show an orchestrated circadian rhythm *in vivo*[124] and *in vitro*.[144] On the basis of 2-DG studies and electrophysiological analyses of the neonatal SCN *in vitro*, several investigators have arrived at the conclusion that the SCN manifests circadian rhythms prior to birth. Circadian rhythms have been inferred to occur by embryonic day E19 in rats, several days before birth. Synaptogenesis in the SCN occurs largely postnatally; at E19 there are fewer synapses than cells,[99] and the synaptic antigen synapsin is virtually absent from the SCN between E19 and E21. This makes it unlikely that the circadian rhythms of embryonic SCN are orchestrated by conventional synaptic interaction. Whether the intercellular signals in the embryonic SCN that underlie circadian rhythm orchestration are similar to those in the adult is unclear. In the embryonic SCN two possibilities for communication that merit experimental attention are non-synaptic release of an unidentified neuroactive substance or the presence of gap junctions. Transient expression of gap junctions is common between neurons during certain stages of early development in the cortex[86,183] and locus coeruleus.[25]

Studies of blinded or SCN-lesioned mother rats and their neonatal offspring have revealed that the SCN of embryonic rats is entrained to the mother's rhythm.[179] The mechanism of fetal to mother entrainment is not clear. One intriguing hypothesis is that melatonin released by the mother's pineal gland could entrain fetal SCN. The SCN has melatonin receptors and a pineal melatonin circadian rhythm exists. Melatonin administration can be used to entrain the fetal SCN to an external rhythm.[31] Although melatonin appears sufficient to entrain the neonatal SCN, it is not a necessary agent, as the SCN

of embryonic rodents remains entrained to their mother's SCN-regulated rhythm even after maternal pinealectomy.[125] Similarly, surgical removal of several endocrine organs is without apparent effect on the entrainment of fetal SCN to the mother's rhythm.[126] It has not been possible to measure a behavioral rhythm in the embryonic or young neonatal rodent, and 2-DG studies are based on a single determination of uptake after brain removal. These studies, therefore, are based on indirect evidence for SCN rhythmicity on the basis of analysis of SCN from groups of rats and extrapolation from values obtained relative to coherence of litter values from mothers from different experimental conditions. *In vitro* electrophysiological and cytochemical analyses of the embryonic SCN may facilitate determination of which factors might orchestrate cellular behavior prior to synaptogenesis.

What cellular mechanisms do 2-DG uptake studies reveal, particularly those found in embryonic animals? Radioactive 2-DG is used as an index of relative energy utilization in different regions of the brain. 2-DG uptake may represent neuronal activity, but the dominant source of energy consumption appears to be the plasmalemma pumps that maintain ion homeostasis in the intracellular and extracellular compartments. Action potentials and synaptic potentials are due to changes in the membrane conductances and are independent of metabolic energy once the ionic gradients have been established.[58] On the other hand, intense synaptic and action potential activity in the nervous system requires energy consumption to maintain ionic gradients, but this intense synaptic activity is not found in the embryonic SCN. In rodents, 2-DG levels are high in the SCN in the day, and low at night compared to the surrounding hypothalamus.[138] It is possible that the circadian rhythm of 2-DG uptake is a glial phenomenon as much as a neuronal one, although astrocytes, like neurons, are not mature in the embryonic/neonatal SCN. A general overview of factors underlying 2-DG uptake in the SCN is presented elsewhere.[137] The subcellular localization of the 2-DG has not been determined in the SCN. That circadian rhythms of 2-DG uptake are also found *in vitro* suggests that the daily change in 2-DG uptake is not due to the presence of, or uptake by, afferent axons innervating the SCN from distant neuronal loci. The 2-DG studies in fetal and neonatal tissue provide additional support for the hypothesis that mechanisms other than axonal conduction and synaptic transmission can mediate synchronization of the activity of SCN cells.

7. THE COLD CLOCK

Cells of the circadian clock appear to function even under conditions well below normal mammalian body temperature, suggesting cellular interactions that are temperature compensated or that are not particularly temperature sensitive. Several experiments have demonstrated that even when the body temperature of a ground squirrel in hibernation dropped by over 20 C from its normal euthermic temperature to a colder one of about 12 C, circadian fluctuations in body core temperature can still be detected.[45] Of interest, each bout of hibernation which lasted about one week exhibited a tightly regulated body temperature rhythm with a circadian period that appeared reset with a slightly different period length with each successive bout of hibernation. Finally, the length of the hibernation bout appeared to be some multiple of the period of the circadian clock, supporting the concept that the circadian clock operates at cold temperatures.[45,156] Similar observations have been reported for hibernating bats.[92] In spite of the cold brain temperature, the squirrel SCN shows a relatively high 2-DG uptake during hibernation compared with other brain regions.[68] Taken together, these data suggest that at temperatures which would have severe disruptive effects on normal mammalian neuronal electrical activity and transmitter release, cells in the circadian clock still appear to communicate and keep time.

8. SUPRACHIASMATIC NUCLEUS OUTPUT

8.1. *Efferent axons*

Axonal projections from the SCN terminate both within the SCN[166] and project to nearby hypothalamic regions, including the periventricular and preoptic areas, and the paraventricular, ventromedial and dorsomedial nuclei.[153,177] Based on ultrastructural analysis, many SCN axons have a very small diameter, suggesting that they conduct action potentials slowly, making them difficult to detect with the light microscope even after cytochemical labeling. A particularly dense projection has been described from the SCN to the subparaventricular region of the hypothalamus, a region with widespread connections throughout the limbic system.[177,178] SCN extrahypothalamic projections have been reported in the midline thalamus, particularly the paraventricular and paratenial thalamic nuclei, thalamic intergeniculate leaflet, midbrain, and the intermediate lateral septal nucleus.[9,78,151,153,159] As discussed earlier, most of the neuronal cell bodies in the SCN contain GABA, suggesting that a major part of the message conveyed to other regions of the brain from the SCN is inhibitory.

8.2. *Suprachiasmatic nucleus transplantation*

In recent years, considerable progress has been made on transplantation of cells into the brain. One of the most exciting directions in this area is the transplantation of a functional clock into arrhythmic animals. These transplantation studies have raised some interesting questions about the nature of the efferent signal released by SCN cells, which drives

neurons in other regions of the brain and underlies circadian rhythms in general. After electrolytic lesions of the SCN, animals appear to permanently lose their ability to express behavioral or endocrine circadian rhythms.[100,155,171] However, if the SCN area from a neonatal rodent is transplanted into a lesioned animal, circadian rhythms return. This finding has been repeated by many laboratories.[33,35,80,121,130,132,149] That the rhythm is necessarily generated by the transplanted tissue itself, rather than by the tissue simply supplying some intermediate signal, is shown by experiments where the SCN of a mutant short-period hamster is transplanted into a normal SCN-lesioned hamster, and the host adopts the short circadian period of the mutant donor animal encoded by the transplanted tissue.[121]

Several phenomena related to these experiments merit considerable interest. First, the rhythm sometimes appears to return before any massive axonal outgrowth from the transplant can be demonstrated. In fact, some animals were reported to show a recovered rhythm as early as four days after the transplant.[80] Another observation relates to the neuroanatomical site of the transplant. Most investigations show the location of the area receiving the SCN implant which gives the most robust recovery of function as being in the general area of the SCN, or the third ventricle between the normal location of the bilateral SCN.[33] However, some reports suggest recovery of function even if the SCN is transplanted to a site some distance from the SCN, for instance the thalamus.[130,149] Finally, some evidence exists for recovery of circadian function after disaggregating SCN cells. Vasopressin circadian rhythms have been reported in cultures of cells dissociated prior to plating.[105] Circadian behavioral recovery after transplantation of dispersed SCN cells into an SCN-lesioned hamster has also been reported.[149]

SCN circadian rhythm transplant work is particularly demanding. It rests first on the assumption that the entire SCN is completely destroyed prior to the implantation of new tissue. This is important since a recovery of function after partial SCN lesions could be due to small regions of the SCN not completely eliminated by the lesion.[100,171] This is a crucial consideration, because if there is a possibility for recovery of circadian function in the absence of an implant, the recovery following a transplant might incorrectly be attributed to the SCN transplant. Animals must be followed over long periods of time, and tracked continuously, generally with running wheels and electronic recording devices. Implanted tissue must be identified as containing at least some SCN. This is often done with immunostaining for neuroactive substances found in the SCN but with limited expression outside the SCN, for instance VIP or vasopressin. Given the problems associated with the multi-dimensional and complex results, the use of short-period mutant rodents[122] as SCN donors will reduce difficulties in interpretation due to the recovery of a circadian rhythm with a different period than the animal's previous period length.

One explanation for the transplant data is that the SCN is releasing a diffusible substance which even in small amounts is sufficient to influence other neurons in the medial subparaventricular hypothalamus, the site of termination of many SCN efferent axons.[178] This agent would probably be an uncommon agent, as the extracellular space may have a baseline level of many neuroactive agents, and a common transmitter might get lost in the "noise" of other transmitters. The possibility of widespread diffusion of transmitters to receptors in locations removed from axonal terminals has been addressed in a number of systems throughout the brain.[56] The SCN "hormonal hypothesis" is not totally consistent, however, with experiments suggesting that knife cuts around the SCN which cut the outgoing axons result in permanent abolition of circadian rhythms.[61,100,109,111,154,171] If the hormonal hypothesis were correct, one might expect that the remaining axon terminals proximal to the axon damage would eventually release the "clock hormone", which would lead to a recovery of function. This type of recovery has received little experimental support;[53] the lack of recovery after knife cuts could be explained by a further hypothesis that neuroactive substances are not released from the cut axons of adult SCN or cannot diffuse into the correct target area in the more caudal medial hypothalamus. This, however, contrasts with a small number of cases suggesting that the transplant can be located some distance from the main efferent target zone of the SCN in the hypothalamus and still restore circadian function in an SCN-lesioned animal.[130,149]

If the "clock message" is hormonal, could it be GABA, particularly given the presence of GABA in most SCN cells and half of all SCN presynaptic boutons? Although the SCN efferent signal at synaptic junctions may well be GABA, it seems unlikely that GABA could serve as a diffusible signal. Fifty per cent of all the presynaptic terminals in all the regions of the hypothalamus we examined were immunoreactive for GABA,[32] and it appears implausible that GABA released from relatively few axons at any distance from a target neuron would have any informational value in a sea of axons releasing GABA and astrocytes taking up GABA. Similarly, identified peptides synthesized in the SCN may not be as widespread as GABA, but may still be found in many other hypothalamic and non-hypothalamic brain regions in addition to the SCN.

9. OVERVIEW

Circadian rhythms are such a pervasive factor in the lives of all animals, from mammals to unicellular organisms, that one might expect multiple means of intercellular integration and communication. Redun-

dant mechanisms may underlie cellular communication within the SCN and message communication to other parts of the brain. Thus, the cellular behavior of adult SCN neurons may be orchestrated by mechanisms different from, in addition to, or similar to those that drive embryonic rhythms. Future studies addressing the membrane biophysics of SCN perikarya, dendrites, and axons, the physiology of SCN astrocytes, the nature of diffusible chemical signals in the SCN, and the rhythms of single cells and groups of cells *in vivo* and *in vitro* should add greater insight into the intercellular communication of the SCN and the role of this hypothalamic nucleus as a circadian clock.

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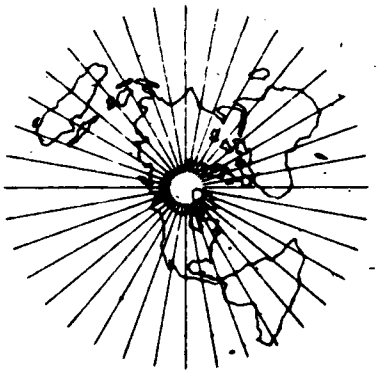

ABSTRACTS

World Federation of Sleep Research Societies

Australasian Sleep Association
Canadian Sleep Society
European Sleep Research Society
Japanese Society of Sleep Research
Latin American Sleep Society
Sleep Research Society (United States)

NON-CHEMICAL SYNAPTIC MECHANISMS SYNCHRONIZE NEURONAL ACTIVITY IN THE SUPRACHIASMATIC NUCLEUS (SCN)

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The SCN, which contains the mammalian biological clock, exhibits a circadian rhythm of firing rate. Since these data are derived from neuronal populations they also imply that neuronal activity in the SCN is synchronized. To examine possible mechanisms of synchronization, coronal hypothalamic slices (350-500 μm) were prepared 2 h before the dark phase (12:12 L:D) from male rats. Simultaneous multiple-unit recordings showed that the circadian rhythm of neuronal activity level was correlated between different locations within the SCN ($0.63<r<0.99$, $p<0.0015$) and thus confirmed the neuronal *synchronization* (n=6). To study the mechanisms of synchronization in the SCN, extracellular Ca^{2+} was replaced with Mg^{2+} (+0.1 mM BAPTA), which blocks chemical synaptic transmission and increases membrane excitability. This treatment produced periodic bursts of action potentials which were synchronized throughout the SCN (n=11), but were not synchronized with similar bursts in the contralateral SCN (n=9). Simultaneous recordings of single and multiple-unit activity revealed that 57% of the neurons (n=23) fired action potential(s) during the burst with variable discharge patterns (see Fig. 1). Furthermore, a mixture of N-methyl-D-aspartic acid (NMDA), non-NMDA and γ-amino-butyric acid type A ($GABA_A$) receptor antagonists [DL-2-amino-5-phosphonopentanoic acid (AP-5) - 100 μM, 6,7-dinitroquinoxaline-2,3-dione (DNQX) - 50 μM and bicuculline - 50 μM] had no effect on burst synchrony (n=6). Whole-cell patch-clamp recordings confirmed that the Ca^{2+}-free medium blocked evoked postsynaptic potentials (PSPs) (n=4) and that the mixture of antagonists blocked the remaining spontaneous PSPs (n=6). These results indicate that a loose type of neuronal synchronization can occur in the SCN without active chemical synapses, which suggests that a different mechanism of communication exists. A similar mechanism(s) of neuronal synchronization may coordinate the cellular elements in the SCN responsible for circadian rhythms in mammals.

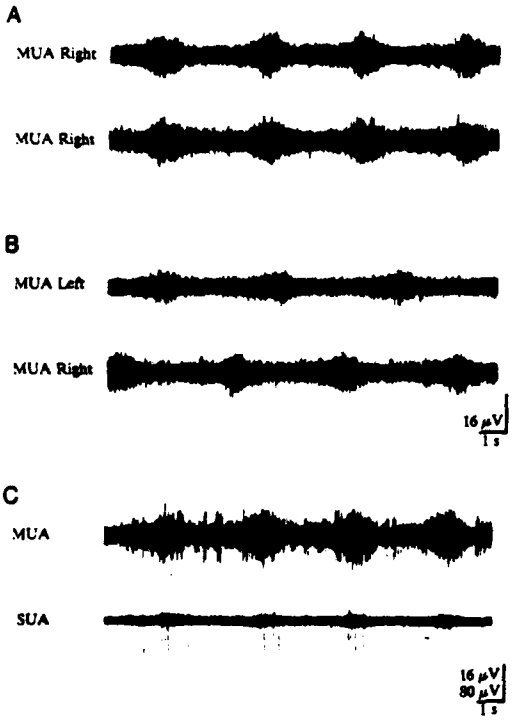

Fig. 1. Simultaneous extracellular recordings during bursting activity in Ca^{2+}-free solution. (A) Simultaneous recordings of multiple-unit activity (MUA) from two locations in the same SCN (right side) indicate synchronous bursts. Interburst interval gradually increased from 1 s to a maximum of up to 10 s after several hours and the burst duration ranged from 0.5 to 2.0 s. (B) Simultaneous recordings of MUA from opposite SCNs (left and right) indicate that the bursts were not synchronous and had different interburst intervals (recorded in the same slice as A). (C) Single-unit activity (SUA) recording during a MUA burst in the same SCN; the SUA bursts coincided with the MUA bursts.

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WHOLE-CELL PATCH-CLAMP ANALYSIS OF NEURONAL EXCITABILITY IN THE SUPRACHIASMATIC NUCLEUS (SCN).

Y. Bouskila*[1,2] and F. E. Dudek[2]. [1]Interdept. Neurosci. Grad. Prog., UCLA Sch. of Med., Los Angeles, CA 90024 and [2]Dept. of Anatomy & Neurobiol., Colorado State Univ., Fort Collins, CO 80523.

The neurons of the SCN exhibit a circadian rhythm in firing rate which may result from modulation of their excitability. To examine neuronal excitability and membrane properties, coronal hypothalamic slices (400 μm) were prepared and neurons were recorded in the whole-cell patch-clamp mode. Spike trains were elicited by graded current pulses (producing 1.6-2.4 mV steps for 200 ms) near spike threshold. Most neurons (n=6) exhibited a high accommodation constant (2.32 ± 0.46, defined as the ratio of last interval to first in a spike train) and had profound spike broadening and spike amplitude reduction (except for one cell). A few neurons (n=2) showed almost no spike accommodation (accommodation constant of 1.28 ± 0.06, $p<0.003$) at all firing rates. The accommodating neurons differed from the non-accommodating neurons by their lower whole-cell capacitance and input resistance. It is unlikely that these differences are due to recording quality since the two cell groups were not significantly different in first spike amplitude, half-peak duration and threshold, resting membrane potential or slope of current-frequency (I-F) relation. Spike accomodation may be important in modulating neuronal excitability and it should now be possible to identify the underlying currents. Supported by AFOSR.

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LOCAL SYNAPTIC CIRCUITS IN THE RAT SUPRACHIASMATIC NUCLEUS.

G.J. Strecker* and F.E. Dudek. Dept. Anatomy and Neurobiology, Colorado State Univ., Fort Collins, CO 80523.

Previous anatomical studies have indicated that a large fraction of neurons and presynaptic terminals in the suprachiasmatic nucleus (SCN) contain GABA. Furthermore, electrical stimulation of regions outside the SCN has been found to produce GABAergic synaptic potentials in SCN neurons, but it is unclear whether such evoked potentials arise from the stimulation of GABAergic neurons within the SCN, or of fibers originating in other areas. To test whether SCN neurons form local inhibitory synapses among themselves, we applied brief pulses of glutamate (10 mM, 0.2 s) to the SCN during whole-cell voltage-clamp recording in thin (150 μm) hypothalamic slices. Such glutamate pulses would be expected to stimulate predominantly cell bodies in the SCN rather than fibers of passage.

Whole-cell voltage-clamp recordings in SCN from 12 to 18 day-old Lewis rats revealed spontaneous outward currents in all neurons (n = 33), with amplitudes ranging from 5 to 60 pA at holding potentials near 0 mV, and a frequency of occurence ranging from 0.05 to 10 Hz. Bicuculline (10 μM) blocked these events in 9 of 9 neurons, indicating that they were GABA-mediated inhibitory postsynaptic currents (IPSCs). Pressure ejection of glutamate-containing bath solution onto the SCN resulted in clear increases in the rate of IPSCs in 6 of 18 neurons. Spontaneous inward currents, which were presumably excitatory postsynaptic currents (EPSCs), were seen in fewer neurons (n = 11 of 15), possibly due to their lower rate of occurrence (about 0.1 Hz, on average). Glutamate microstimulation did not evoke EPSCs reliably in any of 12 cells. These results suggest that local synaptic cicuits within the SCN exist, and are predominantly inhibitory.

Supported by a grant from the AFOSR.

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